

pulmonary lesion (H14). The third possibility is that the primary lesion may give rise to progressive pulmonary disease either by local extension or bronchogenically. Had those rabbits which exhibited this form of disease been allowed to survive indefinitely it is most likely that they would have succumbed to extensive pulmonary tuberculosis. Hematogenous dissemination occurred in only one instance (H3) and that at an early stage of the disease.

A striking feature in this series of rabbits was the absence of any macroscopical involvement of the hilar glands. With one exception microscopical tubercles in these structures were confined to animals with recent primary lesions in their lungs. Viable bacilli were present before tubercles could be detected and also in one instance (H28) after apparent retrogression of the tubercles. Lurie (1) observed that with bovine infection of naturally resistant and susceptible animals only the latter developed gross disease of their hilar glands. In the rabbit the general level of resistance to human infection is higher than that to bovine, so the present findings support previous ones. Few bacilli could be seen in the glandular lesions and here lies the probable explanation for the paucity of the reaction. In turn this suggests that dissemination by way of the lymphatics from the primary lesion is restricted, as would be expected if resistance to the disease is due basically to localization of the infection to the portal of entry (1).

The development of an undoubted positive reaction to old tuberculin 3 to 4 weeks (on the average) after infection was associated with the persistence of one or more early pulmonary lesions in 8 out of the 9 rabbits sacrificed within the first 12 weeks of the experiment. Of these 9 animals 6 were killed a few days after the tuberculin reaction first became positive. Rabbit H34 was exceptional in that no gross lesion was recognized in the lungs despite a positive tuberculin reaction, the recovery of bacilli from the lungs, and the occurrence of tubercles with viable bacilli in the hilar lymph glands. The most probable explanation is that, despite close scrutiny, a small primary lesion was overlooked, especially as the findings in this animal differ from those in H37 only in the response to tuberculin (Table I). Accordingly a positive tuberculin reaction can be regarded as signifying the development of a pulmonary lesion and, in the latter, caseation is usually, but not necessarily, present. It is thus safe to say that those rabbits whose positive reactions later subsided did have a primary lesion originally and that the absence of gross disease at autopsy implies that such lesion resolved completely or retrogressed sufficiently as not to be visible to the naked eye. Lurie (12) also encountered positive tuberculin reactions in some rabbits exposed to natural contagion with bovine bacilli and in which no evidence of tuberculosis was found at subsequent autopsy. He considered the tuberculin reaction to be specific and to indicate the penetration into, and interaction with the tissues of tubercle bacilli in sufficient concentration to induce an allergic state. It is well recognized that in man, as also in experimental ani-

mals, the tuberculin reaction may wane in intensity as the primary infection retrogresses (13-16) and with this the present findings agree. In the majority of the rabbits in this series, however, waning occurred in association with progressive and stationary tuberculosis. It is impossible to say whether this desensitization was specific or not (15). Lurie (1) observed that resistant animals gave a less intense reaction to tuberculin than susceptible ones after infection with bovine bacilli and this parallels the greater sensitivity to tuberculin of the Negro, who is susceptible to tuberculosis, than of the white, who is more resistant. It would be expected that rabbits, being generally more resistant to human than bovine tuberculosis, would show a less intense tuberculin reaction in the former type of disease than in the latter. This in fact was observed, the reaction being more extensive, indurated, and erythematous as well as sustained, following bovine infection. This phenomenon may be interpreted as meaning that the rabbit localizes the infecting human bacilli more effectively

TABLE V

Relationship of Primary Infecting Dose to the Subsequent Course of the Disease

Estimated dose (approximate)	No. of rabbits showing:			
	Recent Lesions only	Progressive disease	Stationary disease	Retrogressive disease
500	2	3	0	2
1000	3	4	4	0
2000 and over	3	0	1	2

to the site of deposition or that it destroys the bacilli and their products more rapidly and effectively, so hindering the entry of sensitizing antigens into the circulation.

Whether human type tuberculosis in the rabbit progresses or not depends on two possible factors, the dosage of the infecting organism and the natural resistance of the animal. The rabbits in this series were exposed in groups of 4 to 6 and it can be seen from Tables I and II that the estimated infecting dose varied considerably in different animals. In Table V the course of the disease has been arranged according to the dose of bacilli. Within the limits of these experiments dosage does not appear to determine the number of primary lesions which develop nor whether the latter progress or retrogress. In fact, no example of progression occurred with the highest scale of dosage. This contrasts with the previous experience of bovine tuberculosis in which there appears to be a correspondence between the number of organisms estimated as inhaled and the number of bacilli subsequently recovered from the lungs, as well as with the number of primary tubercles engendered and the eventual progress of the disease (1, 19). The conclusion from these experiments must therefore be that

the determining influence on the course of the disease is the degree of natural resistance to the infection.

The occurrence of viable tubercle bacilli in lung tissue apparently devoid of any pathological changes for as long as 15 weeks after infection has a parallel in human pathology. Opie and Aronson (17) recovered by guinea pig inoculation tubercle bacilli from lung tissue unaffected by tuberculosis in twelve out of thirty-one miscellaneous autopsies on individuals dying from causes other than tuberculosis. All twelve cases, however, bore evidence of a previous pulmonary infection as indicated by quiescent fibrocaseous lesions in the lung, pleural scars, or calcified glandular lesions. Later Aronson and Whitney (18) showed that in six of the twelve cases previously reported the tubercle bacilli were all of human type. In three of these six cases the lung tissue was taken from the apex of the lung but in the remainder it came from the base. The viable tubercle bacilli in normal lung tissue may have been derived from the tuberculous lesions in other parts of the same lung and this may be true of the experimental disease, but it is also possible that in the rabbit the organisms recovered from apparently normal lung were deposited at the time of the exposure to infection. Judging by the lesions in the longest survivors these bacilli must eventually die, but the possible significance of repeated exposures at short intervals after a primary one in building up the local concentration of organisms from a subinfective to an infective level must not be overlooked. Clinical experience, especially with children, is in accord with this view.

SUMMARY

1. Pulmonary tuberculosis in unselected rabbits, induced by primary quantitative air-borne infection with human type tubercle bacilli, may retrogress or progress. Some animals whose disease was in a stationary condition might have fallen into one of the above groups had the experiments been prolonged.

2. Within the limits of the observations natural resistance or susceptibility appears to be the chief factor in determining the course of the disease.

3. Following the development of the primary lesions the tuberculin reaction became positive but thereafter proved to be an unreliable indicator of the course of the disease.

4. Tubercle bacilli can be recovered from macroscopically normal lung tissue of rabbits several weeks after primary infection.

5. Reinfection did not induce the formation of new lesions nor alter the course of the disease caused by the primary infection.

I am greatly indebted to Dr. Max B. Lurie for extending to me his advice and the facilities of his laboratory.

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EXPLANATION OF PLATES

All microphotographs were prepared from tissues fixed in formalin and imbedded in paraffin and stained with hematoxylin-eosin.

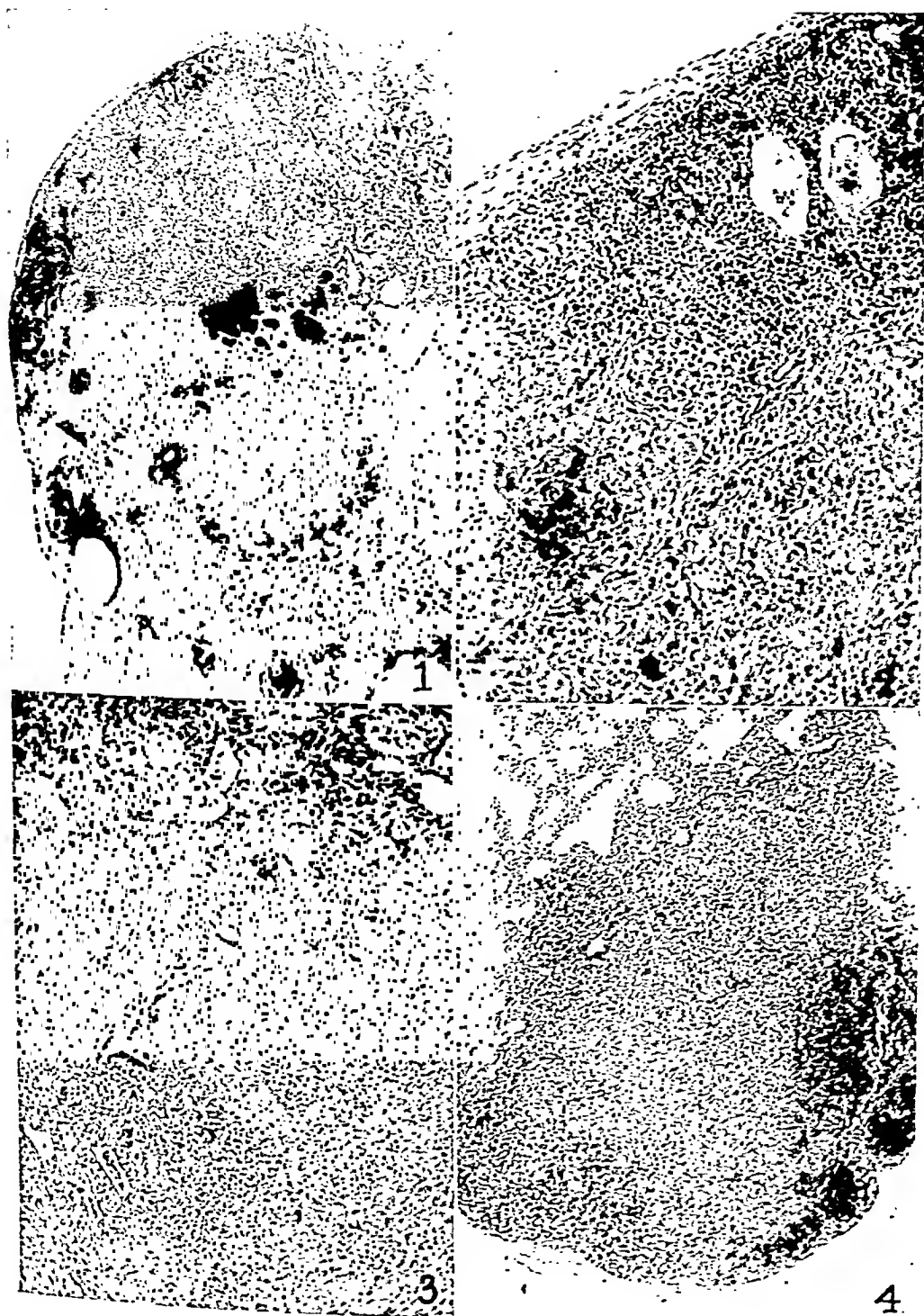
PLATE 30

FIG. 1. Early-primary lesion in rabbit H3, 38 days after a primary infection. Subpleural mass, mainly of epithelioid cells, not very well defined. Central areas of caseation (very dark in photograph). × 24.

FIG. 2. Early primary lesion in rabbit H3, 38 days after a primary infection. Subpleural lymphocytes bordering epithelioid cells with one focus of commencing necrosis. × 145.

FIG. 3. Recent primary lesion in rabbit H17, 61 days after a primary infection. Alveolar metaplasia in the wide inflammatory zone. Epithelioid zone thinned and necrosis more extensive. × 92.

FIG. 4. Hilar lymph node in rabbit H23, 63 days after a primary infection. This pale mass of epithelioid cells with commencing necrosis represents the most advanced degree of change seen in any hilar lymph node. × 46.



(Heppleston: Quantitative air-borne tuberculosis in rabbit)

PLATE 31

FIG. 5. Old lesion in rabbit H31, 129 days after a primary infection. Inflammatory and epithelioid zones now reduced in width. Calcification of the large caseous area prominent. Whole lesion sharply circumscribed. $\times 55$.

FIG. 6. Old lesion in rabbit H15, 207 days after a primary infection. Subpleural fibrosis. Very narrow cellular zone with calcification. Latter more evident in caseous material. $\times 110$.

FIG. 7. Old lesion in rabbit H20, 152 days after a primary infection. Delicate fibrosis of the wall with relatively scanty cellular zone and extensive calcification, especially of the caseous tissue. $\times 150$.



(Hepleston. Quantitative air-borne tuberculosis in rabbit)

THE EFFECT OF ALLOXAN DIABETES ON EXPERIMENTAL CHOLESTEROL ATHEROSCLEROSIS IN THE RABBIT*

I. THE INHIBITION OF EXPERIMENTAL CHOLESTEROL ATHEROSCLEROSIS IN ALLOXAN DIABETES

II. THE EFFECT OF ALLOXAN DIABETES ON THE RETROGRESSION OF EXPERIMENTAL CHOLESTEROL ATHEROSCLEROSIS

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The problem of arterial disease associated with diabetes mellitus has loomed larger and larger ever since the discovery of insulin. However, experimental study of this problem has been thwarted by the fact that dogs and cats, in which experimental diabetes can be readily produced by several methods, are notoriously resistant to the development of arterial disease. On the other hand, although experimental arterial diseases of various types can be produced very easily in rabbits, this species cannot be rendered permanently diabetic by anterior pituitary extracts and its pancreatic tissue, like that of other rodents, has an anatomical distribution such as to render total pancreatectomy an operation of extreme technical difficulty. Thus, prior to the recognition of the diabetogenic properties of alloxan, it was not feasible to study experimentally the effects of diabetes in the one mammalian species that appeared most likely to respond to such a metabolic disturbance with the development of some form of arterial disease. The discovery that rabbits can be rendered permanently diabetic by alloxan has made it possible to embark upon a study of the effects of diabetes on the arteries of rabbits with the hope of gathering experimental data that might have significance not only in relation to the problem of arteriosclerosis developing in man in association with diabetes mellitus but also in relation to the larger problem of arteriosclerosis in general.

The pathological changes encountered in the pancreas and in certain other tissues of animals rendered diabetic by pancreatectomy, by the administration of anterior pituitary extracts, and by the injection of alloxan have been reviewed elsewhere(1). In some of the earlier papers dealing with alloxan diabetes as observed in various species, it was stated that no changes in the arteries were found in association with this experimental disease in spite of the coexistence in some instances of marked

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lipemia, but the duration of these experiments was relatively short. The occurrence of visible lipemia in alloxan diabetes in rabbits lent support to the supposition that atherosclerosis might develop in them after a sufficient period of time, since it is well known that the continued feeding of cholesterol to rabbits is followed by the appearance of lipemia, hypercholesterolemia, and the eventual development of experimental cholesterol atherosclerosis(2-4). Accordingly, Duff and Wilson(5) carried out a series of experiments in which the blood cholesterol levels were followed during the course of prolonged alloxan diabetes in rabbits with a view to determining at the end of the experiments whether any effect had been produced on the arterial system. They found that lipemia was usually an evanescent phenomenon and in some cases did not occur at all. The blood cholesterol was frequently elevated for a time to values of about 350 mg. per cent but it almost always returned to normal in 3 to 6 weeks and so remained for the rest of the animal's life, in spite of the persistence of more or less severe diabetes as judged by the continuance of marked hyperglycemia, glycosuria, polydypsia, polyuria etc. In these experiments they could find no evidence at autopsy of the development of atherosclerosis either in the aorta or other arteries of rabbits that had been diabetic for periods of time ranging from several months up to a maximum of 1 year.

In view of the negative result just described, the experiment reported in Part I of the present communication was undertaken. It was designed to permit of a comparison between the effects of cholesterol feeding in normal rabbits and in rabbits previously rendered diabetic by alloxan. The unexpected result of this experiment was the demonstration that the development of cholesterol atherosclerosis is markedly inhibited in alloxan-diabetic rabbits as compared with non-diabetic control animals, in spite of the fact that hypercholesterolemia of comparable degree is induced in the two groups of animals by cholesterol feeding. This result has already been briefly recorded (6).

The inhibition of the development of experimental cholesterol atherosclerosis in rabbits rendered diabetic by alloxan suggested the importance of determining whether the retrogressive changes that are known to occur in the arterial lesions after the feeding of cholesterol is terminated (2, 3) would be affected by the induction of alloxan diabetes at the completion of an adequate course of cholesterol feeding. An account of an experiment carried out with this end in view is contained in Part II of the present paper.

I. THE INHIBITION OF EXPERIMENTAL CHOLESTEROL ATHEROSCLEROSIS IN ALLOXAN DIABETES

Materials and Methods

The animals employed were young, adult, domestic white rabbits of both sexes. The ages of these animals were unknown. They weighed from 2 to 3 kilos at the beginning of the experiment, and all were healthy, growing animals. Housed in separate metal cages, they were given a diet of Purina rabbit chow and water *ad libitum*. No dietary supplements were used. The animals were divided into convenient groups or series, and after a period of acclimatization of 1 or 2 weeks determinations of the content of sugar in the blood and of

free and total cholesterol in the serum were made in the fasting state by a modification of Folin's micro method, and by the Schoenheimer and Sperry method respectively. After establishing that these quantities were within normal limits, half of the animals were selected at random and were injected with a freshly prepared 5 per cent aqueous solution of alloxan (Eastman Kodak Co.) in the lateral ear vein. The dose was 200 mg. per kilo of body weight. This treatment was followed by the daily injection of 2 to 6 units of protamine zinc insulin and of about 1 gm. of dextrose for a period of 7 to 14 days. The animals were then left without further treatment for a period of 4 to 5 weeks in order to allow for stabilization of the metabolic processes, after which cholesterol feeding was instituted. Urinary sugar and acetone estimations were made when indicated. Fasting blood sugar and cholesterol values were estimated at approximately biweekly intervals.

The normal control and alloxan-injected animals in each series were fed exactly the same doses of cholesterol on the same days of the experiments and, except for the previously mentioned period of insulin and dextrose therapy, were treated exactly alike. The animals of series 3 received dry powdered cholesterol in gelatin capsules, according to the method of Pollak (7). The animals of all other series received a 3.3 or 5 per cent solution of cholesterol in corn oil dissolved and maintained at 60°C. and fed by means of a stomach tube after cooling. The daily dose of cholesterol varied in the different experimental series from 0.25 to 0.75 gm. The details of the total amounts of cholesterol fed, and the durations of feeding in the various experimental series are shown in Table I.

On completion of the period of cholesterol feeding the surviving animals were killed by air embolism, complete autopsies were performed, and the tissues were fixed and sectioned. The aorta and heart were removed *en bloc*, fixed in formalin, stained in Sudan IV, and the fatty deposits in the intima revealed by this method were recorded on standardized, schematic drawings of the aorta. The aorta was then severed from the heart, small blocks were removed for frozen sections, and the remainder was rolled into a coil and embedded in paraffin. These blocks were sectioned in such a manner that a single microscopic section included the entire length of the aorta. The paraffin sections were stained with hematoxylin and eosin, Verhoeff-Van Gieson stain for elastic tissue, and Mallory's phosphotungstic acid hematoxylin. Frozen sections of the aorta, liver, spleen, and adrenal gland were examined after staining with Sudan IV.

The maximum values accepted as normal were: blood sugar 160 mg. per cent, total cholesterol 80 mg. per cent, and free cholesterol 35 mg. per cent. An animal was regarded as being diabetic if the fasting blood sugar average was 300 mg. per cent or greater, and if most of the obvious manifestations of alloxan diabetes, such as persistent polyuria, glycosuria, polydipsia, polyphagia, and weight loss were present. In addition, the histological demonstration of the characteristic hydropic changes in the pancreas (8) and the lesion of Armani in the kidney were taken as confirmatory evidence of the existence of a persistent diabetic state. Certain of the animals that had received a diabetogenic dose of alloxan, although they were initially diabetic, reverted to a normal metabolic state by the time that cholesterol feeding was instituted and thereafter showed neither chemical nor obvious evidence of diabetes. Such animals were classed as "alloxan-recovered" (see Table I). The degree of experimental cholesterol atherosclerosis observed in each animal was graded on an arbitrary scale of 0 to 4 as shown in Fig. 1. It is important to note that atherosclerosis of grade 1 severity was recorded even when only a single fleck of lipid deposit was revealed by careful gross examination after Sudan staining of the entire aorta. This gross grading was confirmed by microscopic examination. The amount of sudanophilic, lipid substance in the liver, spleen, and adrenal was graded in a similar arbitrary manner on a scale of 0 to 4 by microscopic examination of stained, frozen sections. A lipemic condition of the blood induced by the diabetic state and/or cholesterol feeding was similarly graded by inspection of the blood

before and after clotting *in vitro*. A lipemic index for each animal was established by averaging the grades of lipemia observed on all the occasions when the blood was sampled.

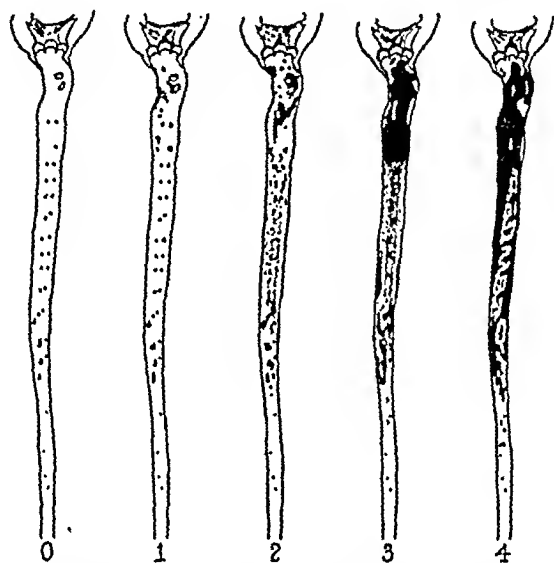


FIG. 1. The standard schematic diagram of the rabbit's aorta used for charting the extent of the atherosclerosis observed at autopsy is illustrated. The five diagrams represent the aortic lesions observed in five animals and show, from left to right, the degrees of atherosclerosis graded as 0, 1, 2, 3, and 4 respectively.

Observations

The observations are summarized in Table I. Over 100 animals were used in this experiment, but the mortality rate from the use of alloxan and from feeding and other accidents was high. Only the 58 animals that completed an experimental course of satisfactory duration are reported upon. These comprised 18 diabetic and 39 non-diabetic animals. In addition, there was one animal (No. 43) that showed a moderate elevation of the blood sugar level in the postprandial state only, and is referred to in the table as mildly diabetic. The 39 non-diabetic animals comprised 13 rabbits that had recovered from the effects of a diabetogenic dose of alloxan ("alloxan-recovered" group), and 26 normal control animals that were subjected to cholesterol feeding alone. The period of cholesterol feeding varied from 52 to 91 days. The total dose of cholesterol fed varied between 16 and 65 gm. and the average daily dose of cholesterol fed varied from 180 to 750 mg. among the different experimental series. The chemical values given in the table are arithmetical averages of biweekly determinations made during the period of cholesterol feeding and of one determination made immediately before cholesterol feeding was begun. The very high maximum values obtained in some of the animals are not shown, but are, nevertheless, indicated by the average values recorded.

The dosage of cholesterol and the duration of cholesterol feeding varied from those that induced neither a hypercholesterolemia nor cholesterol atherosclerosis among the control animals (series 1 and 2) to those that induced in the controls a very severe degree of both, with the formation of confluent atherosclerotic lesions extending over the entire intimal surface of the aorta and measuring from one to one and one-half times the thickness of the underlying media. However, inspection of the data in Table I reveals that there was a remarkable discrepancy in the degree of atherosclerosis induced in the diabetic animals as compared with that induced in the comparable groups of control rabbits fed exactly the same quantities of cholesterol within the same period of time (Figs. 2 and 3). In each experimental group, with the exception of series 1 and 2 in which no atherosclerosis was induced in any of the animals, the diabetic rabbits showed a striking resistance to the induction of experimental cholesterol atherosclerosis of the aorta. On the other hand, the control rabbits and the "alloxan-recovered" group presented the expected incidence and degree of cholesterol atherosclerosis without distinction. The 23 control animals contained in series 3 to 11 inclusive, all presented more or less severe atherosclerosis of the aorta. Of these 23 animals, 8 presented aortic atherosclerosis of grade 1 severity, 4 of grade 2, 4 of grade 3, and 7 of grade 4. The 12 "alloxan-recovered" animals in these same series presented similar findings. Of these 12 animals 1 showed no atherosclerosis, 2 had aortic lesions of grade 1 severity, 4 of grade 3, and 5 of grade 4. In contrast to these results, the 13 diabetic animals in the same groups presented no atherosclerosis in 6 animals, grade 1 aortic lesions (usually minimal) in 6, and aortic atherosclerosis of grade 2 (minimal) in only 1 rabbit. The quantities of sudanophilic lipid material present in the liver, spleen, and adrenals, in general, corresponded closely with that found in the aorta. The diabetic rabbits showed evidence of the same resistance to lipid deposit in the liver, spleen, and adrenals as in the aorta. Indeed, some of the diabetic rabbits were completely protected from the deposition of cholesterol, as judged by careful morphological examination, not only in the aorta but also in these other organs.

Most important is the observation that the diabetic state did not inhibit the development of a hypercholesterolemia that was as high as that induced in the corresponding "alloxan-recovered" and control animals and that was occasionally considerably higher (Figs. 2 and 3). This was also true of both the free and ester fractions of the serum cholesterol; there was no significant alteration in the ratio of ester to total cholesterol content. In addition to exhibiting an equally marked degree of hypercholesterolemia, the diabetic rabbits showed a visible lipemia *in vitro* that was much more marked than that observed in the corresponding non-diabetic animals. The control and "alloxan-recovered" animals did not show more than a moderate to marked opalescence of the serum, while the diabetic animals exhibited a lipemia that was manifest as a milky or creamy appearance that rendered the serum opaque. A further difference in the lipemia of the diabetic and non-diabetic animals was the rapidity with which

TABLE I
Summary of Experimental Data

Series	No.	Sex	Experimental type*	Duration of cholesterol feeding	Total dose of cholesterol	Weight at beginning of cholesterol feeding	Weight at completion of cholesterol feeding	Average blood sugar	Average free serum cholesterol	Average total serum cholesterol	Lipemic index (0-4)	Grade of atherosclerosis (0-4)
				days	gm.	kg.	kg.	mg. per cent	mg. per cent	mg. per cent		
1	1	M	D	91	16	2.98	2.61	508	68	114	0	0
	2	M	D			2.12	1.89	583	89	111	0	0
	3	M	D			2.74	2.92	397	13	32	0	0
	4	M	C			2.56	3.20	109	11	30	0	0
	5	M	AR			3.08	3.51	108	14	32	0	0
2	6	M	D	90	31	2.53	2.24	501	51	71	0	0
	7	M	D			2.09	2.50	473	20	42	0	0
	8	M	C			3.26	4.66	116	11	23	0	0
	9	M	C			3.09	3.75	115	24	55	0	0
3	10	F	D	91	38 (dry)	2.45	1.98	517	18	53	0	0
	11	M	D			3.15	2.64	472	45	90	0.8	0
	12	F	C			3.54	4.06	96	25	84	0	1
	13	F	C			3.08	3.36	96	33	130	0	1
	14	M	C			2.84	2.76	100	27	103	0	1
4	15	M	D	52	39	2.23	2.05	329	220	726	2	0
	16	M	C			2.28	2.63	136	41	120	1	1
	17	M	C			2.41	2.07	153	414	1728	1.3	3
	18	M	AR			3.24	3.58	126	14	48	0	0
5	19	M	D	53	40	2.22	1.59	481	219	423	3.75	0
	20	M	D			2.37	1.88	427	101	197	2.75	0
	21	F	C			4.58	5.15	119	64	210	0.5	1
	22	F	C			3.50	4.59	131	105	316	1	1
	23	F	C			3.02	3.72	129	111	389	1.5	2
	24	M	AR			3.32	3.63	111	32	77	0.25	1
6	25	M	D	90	46	2.58	1.84	355	192	396	3.7	1
	26	F	D			2.73	2.60	354	57	159	1.7	1
	27	F	C			3.90	4.14	114	155	334	1.7	4
	28	F	C			3.62	4.40	123	139	349	2.0	4
	29	M	AR			3.04	3.23	116	104	359	1.8	4
	30	M	AR			3.43	3.78	108	89	232	1.2	4
	31	F	AR			3.55	3.98	115	100	367	1.8	4
7	32	M	D	89	48.5	3.48	3.45	452	99	277	2	1
	33	M	C			4.38	5.25	114	15	63	0	2
	34	M	C			3.40	3.68	118	66	167	1.4	3
	35	M	C			3.29	3.13	111	97	316	1.8	4
	36	M	AR			4.45	4.99	110	59	140	1	3
	37	M	AR			3.94	4.31	116	60	123	1	3

TABLE I—*Concluded*

Series	No.	Sex	Experimental type*	Duration of cholesterol feeding	Total dose cholesterol	Weight at beginning of cholesterol feeding	Weight at completion of cholesterol feeding	Average blood sugar	Average free serum cholesterol	Average total serum cholesterol	Lipemic Index (0-4)	Grade of atherosclerosis (0-4)
				days	gm.	kg.	kg.	mg. per cent	mg. per cent	mg. per cent		
8	38	M	D	76	52	2.47	2.89	375	171	401	4	0
	39	M	C			3.50	4.06	113	121	425	1.75	4
	40	M	C			3.72	4.09	118	70	272	1.25	2
	41	M	AR			3.66	4.23	121	98	340	1.5	3
	42	M	AR			3.12	—	136	159	472	2.25	4
	43	M	MD			3.70	4.00	147	120	309	1.0	1
9	44	M	D	82	52	2.96	3.36	335	459	1970	1.6	2
	45	M	C			2.06	2.83	145	344	632	1.25	1
	46	M	C			2.49	4.10	137	179	334	0.75	3
	47	M	C			2.34	4.17	136	191	491	1.25	4
	48	M	AR			2.94	3.11	177	612	1849	1.5	4
	49	M	AR			3.57	4.50	127	75	357	0.8	1
10	50	M	D	82	60	3.07	3.38	444	505	1075	3.8	1
	51	M	D			3.10	2.19	398	315	703	3.2	1
	52	F	C			4.05	4.20	126	164	562	1.6	4
	53	F	C			3.73	4.51	117	192	602	1.6	2
	54	F	AR			3.85	5.09	115	235	656	2.0	3
11	55	F	D	89	65	2.69	3.31	392	135	346	1.2	1
	56	M	C			2.80	4.17	124	55	221	0	1
	57	M	C			3.17	4.36	128	123	450	1	3
	58	M	C			3.50	4.51	122	143	593	1.3	4

* D = diabetic; C = control; AR = "alloxan-recovered;" MD = mild diabetic.

this phenomenon became apparent after the blood was drawn. Lipemia was apparent in the blood of diabetic animals within 1 to 20 minutes. It was seldom seen in the blood of comparable non-diabetic animals before 30 to 60 minutes after the blood was drawn.

While the data considered above are consistent, the variability of the cholesterol dosage and of the duration of the experiments precludes a detailed analysis of the possible influence of certain other experimental variables, such as sex and change in weight of the animals during the experiment. It is interesting, therefore, to compare the data of individual animals that were similar in as many respects as possible, in order to assess the importance of these factors. The protocols of a number of such animals are given below.

The following protocols are of a diabetic rabbit and of a non-diabetic control

animal that are comparable as to sex and in all other respects, except that in the diabetic animal the degree of lipemia was much greater, and the amount of lipid

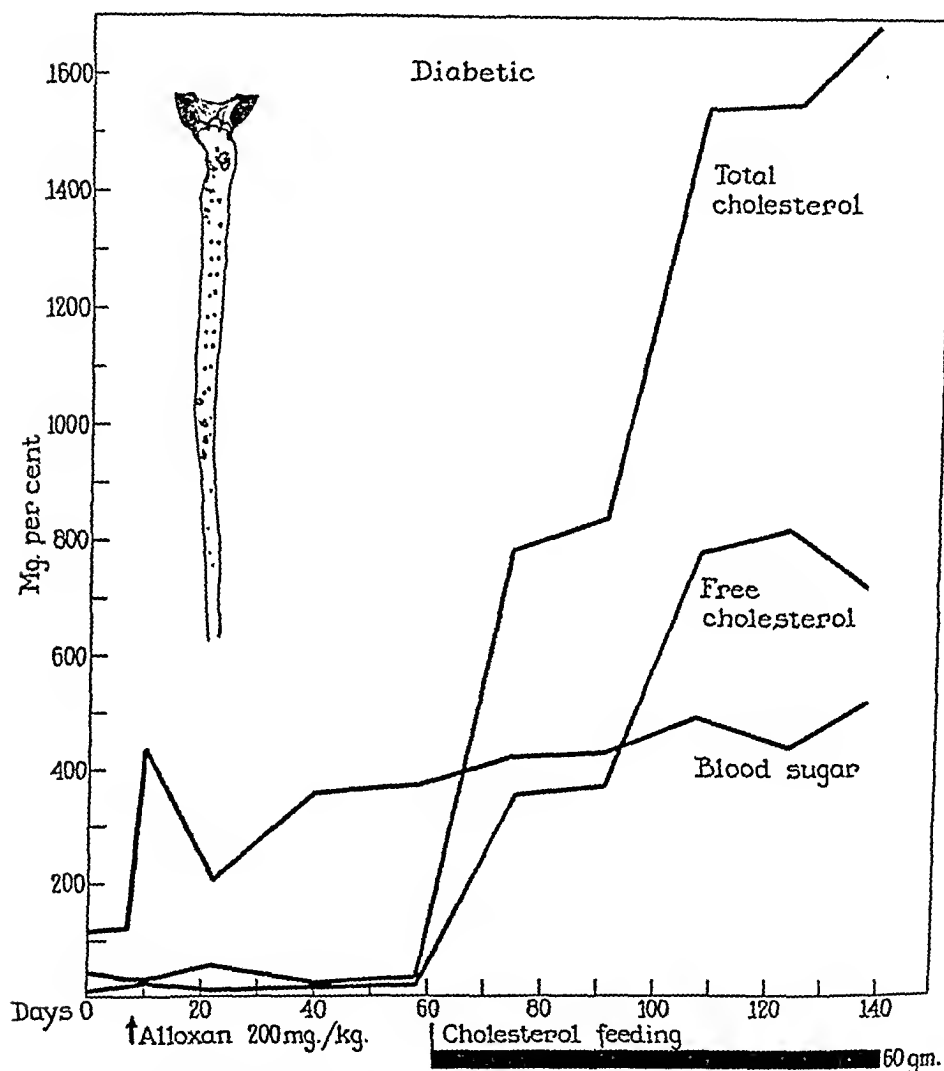


FIG. 2. Diagram and graph illustrating the procedures and findings in diabetic rabbit 50, series 10. The aortic atherosclerosis shown was recorded as grade 1.

deposition in the aorta, liver, spleen, and adrenal was much less than in the control rabbit.

No. 38. Diabetic. Male.—Dose of cholesterol per day, 0.68 gm. Duration of feeding, 76 days. Average total serum cholesterol, 401 mg. per cent. Lipemic index, 4.0. Ather-

osclerosis, grade 0. Liver fat, grade 0. Splenic fat, grade 1. Adrenal fat, normal. Weight gain, 0.42 kilo.

No. 39. *Control. Male.*—Dose of cholesterol per day, 0.68 gm. Duration of feeding, 76 days. Average total serum cholesterol, 425 mg. per cent. Lipemic index, 1.75. Ather-

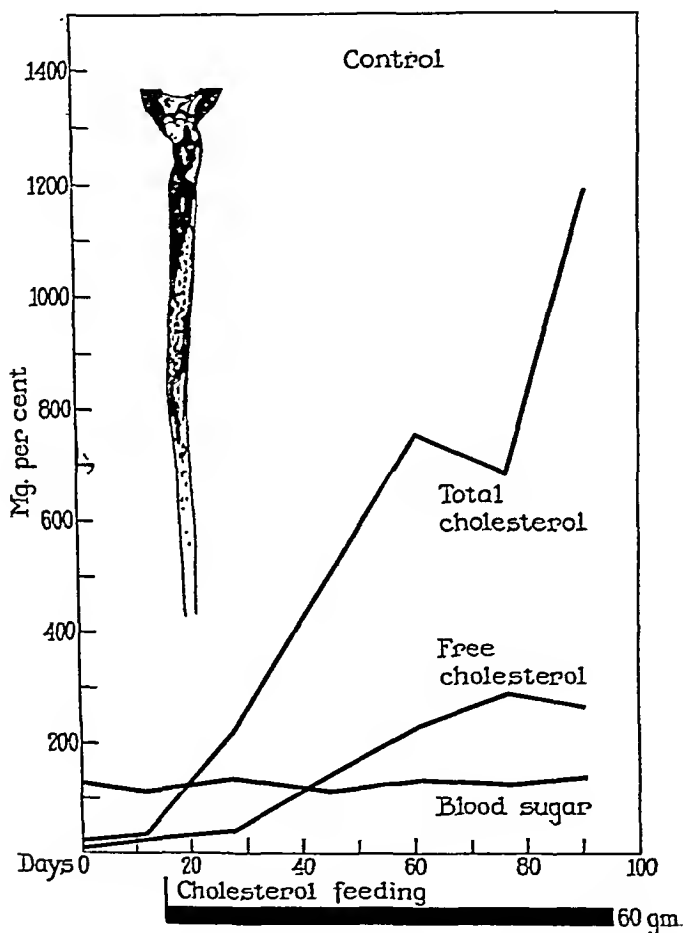


FIG. 3. Diagram and graph illustrating the procedures and findings in non-diabetic control rabbit 52, series 10. The aortic atherosclerosis shown was recorded as grade 4.

osclerosis, grade 4. Liver fat, grade 4. Splenic fat, grade 3. Adrenal fat, grade 4. Weight gain, 0.56 kilo.

The following two groups of protocols in which the comparable diabetic, "alloxan-recovered," and non-diabetic control animals alternate in sex, also indicate that sex is not the determining factor in inhibiting the deposition of lipids in the aorta and elsewhere.

No. 26. *Diabetic. Female.*—Dose of cholesterol per day, 0.52 gm. Duration of feeding, 90 days. Average total serum cholesterol, 159 mg. per cent. Lipemic index, 1.7. Atherosclerosis, grade 1. Liver fat, grade 1. Splenic fat, grade 1. Adrenal fat, grade 1. Weight loss, 0.13 kilo.

No. 36. "*Alloxan-Recovered.*" *Male.*—Dose of cholesterol per day, 0.54 gm. Duration of feeding, 89 days. Average total serum cholesterol, 140 mg. per cent. Lipemic index, 1. Atherosclerosis, grade 3. Liver fat, grade 3. Splenic fat, grade 3. Adrenal fat, grade 3. Weight gain, 0.54 kilo.

No. 34. *Male.*—Dose of cholesterol per day, 0.54 gm. Duration of feeding, 89 days. Average total serum cholesterol, 167 mg. per cent. Lipemic index, 1.4. Atherosclerosis, grade 3. Liver fat, grade 2. Splenic fat, grade 0. Adrenal fat, grade 2. Weight gain, 0.28 kilo.

No. 25. *Diabetic. Male.*—Dose of cholesterol per day, 0.52 gm. Duration of feeding, 90 days. Average total serum cholesterol, 396 mg. per cent. Lipemic index, 3.7. Atherosclerosis, grade 1. Liver fat, grade 1. Splenic fat, grade 1. Adrenal fat, normal. Weight loss, 0.74 kilo.

No. 29. "*Alloxan-Recovered.*" *Male.*—Dose of cholesterol per day, 0.52 gm. Duration of feeding, 90 days. Average total serum cholesterol, 359 mg. per cent. Lipemic index, 1.8. Atherosclerosis, grade 4. Liver fat, grade 4. Splenic fat, grade 3. Adrenal fat, grade 4. Weight gain, 0.19 kilo.

No. 31. "*Alloxan-Recovered.*" *Female.*—Dose of cholesterol per day, 0.52 gm. Duration of feeding, 90 days. Average total serum cholesterol, 367 mg. per cent. Lipemic index, 1.8. Atherosclerosis, grade 4. Liver fat, grade 1. Splenic fat, grade 0. Adrenal fat, grade 2. Weight gain, 0.43 kilo.

No. 27. *Control. Female.*—Dose of cholesterol per day, 0.52 gm. Duration of feeding, 90 days. Average total serum cholesterol, 334 mg. per cent. Lipemic index, 1.7. Atherosclerosis, grade 4. Liver fat, grade 2. Splenic fat, ? Adrenal fat, grade 2. Weight gain, 0.24 kilo.

No. 28. *Control. Female.*—Dose of cholesterol per day, 0.52 gm. Duration of feeding, 90 days. Average total serum cholesterol, 349 mg. per cent. Lipemic index, 2. Atherosclerosis, grade 4. Liver fat, grade 1. Splenic fat, grade 1. Adrenal fat, grade 2. Weight gain, 0.78 kilo.

Inasmuch as equal daily doses of cholesterol were fed to rabbits of different body weights, it is conceivable that the apparent inhibitory effect of the diabetic state might have occurred because of the chance feeding to the non-diabetic animals of a higher dose of cholesterol per kilo of body weight. In the following protocols the diabetic and non-diabetic animals are matched as to sex, duration of cholesterol feeding, average total serum cholesterol, and, in addition, as to dosage of cholesterol calculated as an average dose per day per kilo of average body weight during the feeding period. It is apparent from examination of these protocols that the experimental result was not determined by actual or relative differences in the dosages of cholesterol.

No. 11. *Diabetic. Male.*—Dose of cholesterol (dry) per day per kilo, 145 mg. Duration of feeding, 91 days. Average total serum cholesterol, 90 mg. per cent. Lipemic index, 0.8. Atherosclerosis, grade 0. Liver fat, grade 0. Splenic fat, grade 1. Adrenal fat, normal. Weight loss, 0.51 kilo.

No. 14. *Control. Male.*—Dose of cholesterol (dry) per day per kilo, 150 mg. Duration

of feeding, 91 days. Average total serum cholesterol, 103 mg. per cent. Lipemic index, 0. Atherosclerosis, grade 1. Liver fat, grade 0. Splenic fat, grade 0. Adrenal fat, normal. Weight loss, 0.08 kilo.

No. 44. Diabetic. Male.—Dose of cholesterol per day per kilo, 203 mg. Duration of feeding, 82 days. Average total serum cholesterol, 1970 mg. per cent. Lipemic index, 1.6. Atherosclerosis, grade 2. Liver fat, grade 2. Splenic fat, grade 3. Adrenal fat, grade 2. Weight gain, 0.40 kilo.

No. 48. "Alloxan-Recovered." Male.—Dose of cholesterol per day per kilo, 201 mg. Duration of feeding, 82 days. Average total serum cholesterol, 1849 mg. per cent. Lipemic index, 1.5. Atherosclerosis, grade 4. Liver fat, grade 4. Splenic fat, grade 3. Adrenal fat, grade 4. Weight gain, 0.07 kilo.

Inspection of the data presented in all the groups of protocols given above indicates further that gain or loss of body weight during the course of the feeding period exercised no determining influence on the result of the experiment.

It may be added that it was not possible to select pairs or groups of diabetic and non-diabetic animals matched as in the groups detailed above that yielded evidence contrary to that already presented.

Careful morphological examination, both grossly and microscopically, of the aorta and other organs revealed in varying degrees, as already indicated in Table I, the lesions that have been described by many authors as the characteristic sequelae of prolonged cholesterol feeding in rabbits (2-4). Not only were these lesions characteristic in form and location in the cholesterol-fed control animals, but also in the "alloxan-recovered" and diabetic rabbits. The only distinguishable difference in the morphology of the aortic lesions was a quantitative one as detailed above. This was true also of the lipid deposition in the liver, spleen, and adrenal glands. It should be noted, moreover, that in those parts of the aorta and other arteries that were uninvolved by cholesterol atherosclerosis, no differences could be distinguished microscopically between the diabetic and non-diabetic rabbits. The diabetic animals regularly presented the lesion of Armanni in the kidney and the hydropic changes in the pancreatic islets and ductules that have been described in detail elsewhere as characteristic of prolonged alloxan diabetes (8). The islets of Langerhans and the pancreatic ductules in the "alloxan-recovered" animals lacked hydropic changes but careful study of the islets revealed a peculiar and characteristic disturbance of cell arrangement which was distinctly different from the normal. However, in no other organ, including the thyroid gland, was there any evidence of a histologic difference between the diabetic, "alloxan-recovered," and control animals.

II. THE EFFECT OF ALLOXAN DIABETES ON THE RETROGRESSION OF EXPERIMENTAL CHOLESTEROL ATHEROSCLEROSIS

Materials and Methods

Eighty-one young, adult, domestic white rabbits of both sexes were employed. Of these 32 completed a satisfactory experimental course, and comprised two experimental groups.

Each of the 25 rabbits in one group was fed 61.5 to 62.5 gm. of cholesterol during 88 to 90 days. In a second group of 7 rabbits each was fed 40.5 gm. of cholesterol during 59 days. The individual daily dose of cholesterol was 0.75 gm. fed by means of a stomach tube as a 5 per cent solution in warm corn oil.

Determinations at intervals of approximately 3 weeks were made of the non-fasting blood sugar, and of the free and total cholesterol content of the serum by a modified Folin micro method and the Schoenheimer-Sperry method respectively.

Immediately following the completion of the period of cholesterol feeding about one-half of the animals of each group received an intravenous injection of 150 mg. of alloxan per kilo of body weight administered as a 5 per cent aqueous solution. No insulin or other therapy was employed. The animals were given an unlimited diet of Purina rabbit chow and water *ad libitum*.

The animals, including diabetic, control, and those that recovered from the effects of a diabetogenic dose of alloxan after a brief period of diabetes ("alloxan-recovered"), were sacrificed at intervals of 1 to 16 weeks after the cessation of cholesterol feeding. Complete autopsies were performed and the tissues were treated in the same manner as described in Part I of this paper. The criteria of the diabetic state and the grading of the degree of aortic atherosclerosis and of the amount of lipid deposited in the liver, spleen, and adrenal were also the same as those used in the experiment described in Part I.

Observations

The experimental procedures and observations are summarized in Table II, in which the average values of serum cholesterol given are those obtained during the cholesterol feeding period, including one determination made the day before cholesterol feeding was begun. The blood sugar values are averages obtained after the cessation of cholesterol feeding. Among the animals that were injected with alloxan, the average of the blood sugar content includes one determination made before alloxan was administered. The period of retrogression was that between the cessation of cholesterol feeding and death of the animal. In the diabetic animals the duration of diabetes was the same as the period of retrogression.

The results as shown in Table II did not demonstrate that alloxan diabetes has any effect on the rate or degree of retrogression of experimental cholesterol atherosclerosis in the rabbit as judged by gross morphological examination of the aorta stained *in toto* with Sudan IV. Neither did microscopic examination of the entire length of the aorta reveal any appreciable differences between the atherosclerotic lesions of comparable diabetic and non-diabetic animals. The diabetic state was not found to alter appreciably the rate or degree of disappearance of abnormal lipid deposits in the liver, spleen, and adrenal cortex as judged by microscopic examination of appropriately stained frozen sections. In spite of the fact that in certain of the diabetic animals there occurred shortly after the administration of alloxan a distinct increase in the previously existing hypercholesterolemia, they in common with the other diabetic animals, the control, and "alloxan-recovered" rabbits, showed a return of the blood cholesterol to normal levels within 42 days. The only consistent biochemical differ-

TABLE II
Summary of Experimental Data

Series	No.	Sex	Experimental Type*	Total dose of cholesterol	Time of retrogression	Average blood sugar during retrogression	Average free serum cholesterol	Average total serum cholesterol	Aortic atherosclerosis (0-4)
				gm.	wks.	mg. per cent	mg. per cent	mg. per cent	
1	1	F	D	61.5	1	—	90	277	1
	2	F	D	61.5		570	134	382	4
	3	F	C	61.5		122	174	398	3
2	4	F	D	61.5	3	357	163	471	3
3	5	F	D	61.5	5	384	144	426	2
	6	F	C	61.5		134	161	516	4
	7	F	C	61.5		148	107	291	3
4	8	F	D	61.5	6	319	194	472	4
5	9	F	D	61.5	7	425	96	307	3
	10	M	C	61.5		121	—	—	1
6	11	F	D	62.25	9	353	106	261	2
	12	F	C	61.5		121	140	440	4
7	13	F	MD	61.5	11	286	135	430	3
	14	M	D	61.5		410	218	525	4
	15	M	AR	61.5		138	124	318	2
	16	M	AR	61.5		175	81	208	4
	17	M	AR	61.5		163	163	487	2
8	18	F	D	61.5	16	396	183	479	2
	19	F	D	61.5		488	232	480	2
	20	F	C	61.5		133	—	—	2
	21	M	C	61.5		126	83	298	4
	22	M	C	61.5		120	126	371	4
	23	M	C	61.5		111	88	325	2
	24	F	C	61.5		124	143	382	4
	25	F	C	61.5		127	72	240	3
9	26	F	D	40.5	6	309	140	320	3
	27	F	D	40.5		373	88	262	3
	28	F	C	40.5		130	138	424	4
	29	M	C	40.5		120	41	187	3
	30	F	C	40.5		128	145	452	3
	31	F	C	40.5		125	71	214	2
	32	M	AR	40.5		124	83	270	4

* D = diabetic; C = control; AR = "alloxan-recovered;" MD = mild diabetic.

ence observed between the various groups of rabbits was the hyperglycemic state of the diabetic animals. The only morphological differences found to exist between diabetic and non-diabetic animals were the changes in the pancreas and kidney that are associated with the injection of alloxan or with the development of persistent diabetes.

In both the diabetic and non-diabetic animals there was no gross morphological evidence that any appreciable retrogression of the aortic atherosclerosis had occurred during periods of up to 4 months' duration. However, in animals that survived a period of retrogression of 6 weeks or more there was definite microscopic evidence of the disappearance of lipid deposits from the aorta, splenic arterioles, and other small arteries, from the reticulo-endothelial cells of the liver and spleen, and from the parenchymal cells of the liver and adrenal cortex. In the aorta, the gradual disappearance of lipid material was accompanied by a gradual increase in the number of fibroblastic cells in the atherosclerotic lesions.

DISCUSSION

The observations recorded in Part I of this paper demonstrate clearly that, under the conditions described, there was associated with the presence of alloxan diabetes a marked but incomplete inhibition of the development of experimental cholesterol atherosclerosis in cholesterol-fed rabbits. There was also an inhibition of the deposit of sudanophilic lipid substances in the reticulo-endothelial cells of the liver and spleen and in the parenchymal cells of the liver and adrenal cortex. This inhibition occurred in spite of the induction of a marked degree of hypercholesterolemia in many of the diabetic animals, hypercholesterolemia that was usually as high as, and often higher than, that observed in the corresponding control animals.

This inhibitory effect was apparently dependent neither upon the administration of alloxan *per se* nor upon the short initial period of insulin and dextrose therapy, inasmuch as both the diabetic and "alloxan-recovered" animals received such injections before cholesterol feeding was instituted, but only in the diabetic rabbits was the inhibitory effect apparent. The "alloxan-recovered" animals responded to cholesterol feeding exactly as did the control animals. In addition, it was found that the inhibitory effect was not dependent on the sex or weight of the animal, nor upon the daily dosage of cholesterol, the form in which it was administered, nor the duration of cholesterol feeding. The effect was also independent of changes in body weight occurring during the course of our experiments and of the actual degree of hypercholesterolemia induced by the administration of cholesterol. Moreover, there was no gross or histological evidence of a morphological basis for the inhibitory effect either in the aorta or in the other organs in which it was observed. Indeed, the only observed factors consistently associated with the inhibition of the expected morphological effects of cholesterol feeding were the diabetic state and a degree

of visible lipemia considerably greater than that observed in the control animals. A moderate degree of visible lipemia in the control animals was regularly associated with the development of severe atherosclerosis of the aorta. On the contrary, a marked degree of visible lipemia was observed in a large proportion of the diabetic animals that presented at autopsy only a minimal degree of aortic atherosclerosis.

Objection might possibly be raised to our inference that the injection of alloxan *per se*, apart from its diabetogenic effects, was not responsible for the inhibitory effect observed in the diabetic animals on the ground that the "alloxan-recovered" rabbits did not provide a valid control of this possible factor. It could be argued that the "alloxan-recovered" animals were less susceptible to the general effects of alloxan than the diabetic animals as indicated by the very fact that permanent diabetes failed to develop in them. Animals that fail to respond with the development of permanent diabetes to a dose of alloxan that is diabetogenic to a majority of the species are frequently referred to in the literature rather loosely as "alloxan-resistant." However, in our "alloxan-recovered" animals, the diabetogenic effect of alloxan was manifested initially in the production of a temporary diabetic state of mild or moderate severity from which spontaneous recovery occurred within the period of several weeks before cholesterol feeding was started. Clearly, the injection of alloxan was effective, at least to a degree, but these particular rabbits displayed a capacity for recovery that distinguished them from the permanently diabetic animals.

Although we have referred to these animals as "alloxan-recovered" there is reason to believe that such animals have not returned to a strictly normal state. In our present observations, the "alloxan-recovered" animals during the period of cholesterol feeding showed neither obvious nor chemical evidence of diabetes. However, in other experiments (9) more detailed and precise studies have demonstrated that alimentary glycosuria may be present in such animals in spite of normal fasting blood sugar levels and that this is dependent on the occurrence of slight postprandial hyperglycemia. These residual metabolic defects are correlated with the presence of definite histological alterations in the islets of Langerhans which were detectable in the animals of our present experiments months after the injection of alloxan.

There is ample evidence, therefore, to indicate that our "alloxan-recovered" animals did not tolerate the injection of alloxan without suffering from its effects. Accordingly, we are inclined to regard these animals, at least tentatively, as providing a suitable control of the effects of alloxan injection *per se*. Further experiments currently in progress are designed to settle this point definitely by determining whether the inhibitory effect observed in alloxan-diabetic rabbits is abolished by controlling the diabetic state with insulin.

While we are quite unable to offer a specific explanation of the inhibitory

effect observed in the present experiments, it would appear, in view of the considerations set forth in the preceding paragraphs, that it is dependent upon some undefined factor or factors implicit in, or closely associated with, the diabetic state. That the diabetic condition or factors associated with it exercised an influence on the state and stability of the blood lipids in rabbits fed cholesterol in oil is indicated not only by the development of a marked visible lipemia, but also by the inhibition of lipid deposition in the aorta and elsewhere. Since the deposition of lipids in the intima of arteries is an essential feature of the development of experimental cholesterol atherosclerosis, particular interest attaches to any evidence of an alteration of the physicochemical state of the lipids in the blood plasma (*viz.* excessive lipemia) that coexists with protection from the usual effects on the arteries of rabbits associated with hypercholesterolemia.¹

Almost from the first demonstration of the fact that the feeding of cholesterol is capable of producing atherosclerosis in the arteries of rabbits (10), it was recognized that the development of the arterial lesions is associated with a significant elevation of the cholesterol content of the blood (11). This was confirmed repeatedly by subsequent studies which showed that, in general, the severity of the induced atherosclerosis is correlated with the degree and duration of the induced state of hypercholesterolemia (2-4). It has also been shown that the development of experimental cholesterol atherosclerosis can be inhibited by various modifications of the experimental procedure that prevent the development of the expected degree of hypercholesterolemia (3, 4). On the basis of such data, the concept arose that hypercholesterolemia is the sole factor of importance in the genesis of experimental atherosclerosis. This concept was seriously questioned some years ago by Duff (3) on the basis of the evidence then available and, more recently, certain other investigators have emphasized that factors other than hypercholesterolemia may be important in the development of experimental atherosclerosis, pointing out that animals with comparable levels of induced hypercholesterolemia frequently exhibit widely differing degrees of atherosclerosis (12-15).

The absolute inhibition of the development of experimental cholesterol atherosclerosis in an appreciable number of the diabetic rabbits in our experiments in spite of the presence of marked and prolonged hypercholesterolemia shows clearly that the mere existence of a markedly increased quantity of cholesterol in the circulating blood for a considerable length of time is not in itself capable of causing lesions in the arteries. This conclusion is supported by the demonstration of Steiner (16, 17) and others (18, 19) that the addition of choline to the diet inhibits the development of atherosclerosis in cholesterol-fed

¹ This evidence clearly conflicts with the hypothesis of Moreton (*Science*, 1947, 106, 190; and 1948, 107, 371) which postulates that the determining factor in the development of atherosclerosis is the presence in the circulating blood of lipid particles of large size such as are present in abundance in the grossly milky or creamy serum of hyperlipemic states.

rabbits, though it does not prevent the development of marked hypercholesterolemia (16, 17). It is evident, therefore, that the development of experimental cholesterol atherosclerosis is dependent not only upon the occurrence of hypercholesterolemia *per se* but also upon another essential factor or factors as yet undetermined.

We are fully aware that our experimental observations are at variance with the evidence adduced to show that diabetes mellitus in man promotes the development of arteriosclerosis. A logical resolution of this apparent conflict compels consideration of one or more of the following possibilities. First, the conflict may be consequent on species differences. Second, alloxan diabetes in the rabbit may not be metabolically comparable with diabetes mellitus in man. Third, experimental cholesterol atherosclerosis in rabbits may not be comparable with the type of arterial disease to which diabetic patients are prone. Fourth, the impression that occlusive arterial disease in diabetic patients is dependent upon an excessive development of atherosclerosis of the intima of arteries may be erroneous. Obviously, it is impossible in the present state of knowledge to predict which of the possibilities just mentioned may prove to be correct.

The experimental data presented in Part II of this paper clearly fail to demonstrate any difference in the rate of retrogression of experimental cholesterol atherosclerosis in alloxan-diabetic rabbits as compared with control animals. In our experiments the period of retrogression, *i.e.* the period after the feeding of cholesterol was terminated, was limited to 4 months. Whether experiments with longer periods of retrogression would show any differential effect is impossible to say. More prolonged experiments, however, would be technically difficult because of the excessive mortality from alloxan diabetes of long duration in rabbits.

Our negative results are similar to those reported by other investigators who have attempted to influence the retrogression of experimental cholesterol atherosclerosis by the administration of potassium iodide (20, 21). On the other hand, Steiner (22) some years ago brought forward highly suggestive evidence of the ability of choline to bring about some reabsorption of arterial lesions previously induced by cholesterol feeding. Added to this evidence is the recent report of Morrison and Rossi (23) who have described complete reabsorption of the lesions of experimental cholesterol atherosclerosis in 17 out of 23 rabbits given larger daily doses of choline over a period of 182 days after the cessation of cholesterol feeding.

The observations, already cited relative to the effects of choline on the development and retrogression of experimental cholesterol atherosclerosis, coupled with the results of our own experiments on the effects associated with the presence of alloxan diabetes, permit of interesting deductions regarding the process of lipid accumulation in the walls of the arteries of cholesterol-fed rabbits. If

all the observations are correct and correctly interpreted, it is evident that the process of lipid accumulation must represent the resultant of the effects of two separate and distinct sets of factors, those factors the balance of which hinders or promotes the deposit of lipids in the arterial walls, and another set of factors the balance of which hinders or promotes their removal after they are deposited. This is not a new concept but the means of demonstrating its correctness have not hitherto been available. Since alloxan diabetes (or some associated influence) inhibits the development of experimental cholesterol atherosclerosis but has no noticeable effect on the retrogression of the arterial lesions, it follows that the inhibitory effect must be implemented solely or almost solely by interference with the deposit of lipids. On the other hand, the results of Morrison and Rossi (23) indicate that the administration of choline has a powerful effect in facilitating the removal of lipids already deposited in the arterial wall. The effect of choline in inhibiting the development of experimental cholesterol atherosclerosis, as described by Steiner (17) could, therefore, be due either to interference with the deposition of lipids, or to facilitation of their removal as rapidly as they are deposited or to a combination of both effects. However, if choline facilitates the removal of lipids already deposited in the arterial walls, this is the only effect of choline on experimental atherosclerosis that is susceptible of proof by the types of experiment under consideration here. The mechanisms of interference with the deposit of lipids and of facilitation of their removal remain to be investigated and clarified but the means to do so appear now to be at hand.

It is evident that our experimental results find no direct application to the problem of arterial disease in human diabetes. Nevertheless, if it is true that the accumulation of lipids in the intima of arteries is a central feature of human atherosclerosis, as it appears to be of experimental cholesterol atherosclerosis, then the isolation of the factors governing this fundamental biological process in the experimental animal may be expected to help in elucidating the nature of the same process in man.

SUMMARY

A comparison was made of the effects of cholesterol feeding in normal rabbits and in rabbits rendered persistently diabetic by means of alloxan. In the two groups of animals hypercholesterolemia of comparable degree was induced by the feeding procedure. Nevertheless, the severity of the atherosclerosis of the aorta produced in the diabetic rabbits was much less than in the non-diabetic control animals. Indeed, a large proportion of the diabetic animals presented no atherosclerosis whatever. There was a similar inhibition of the deposit of lipid substances in the liver, spleen, and adrenal glands of the diabetic rabbits.

The inhibition of the development of experimental cholesterol atherosclerosis which was associated with the presence of alloxan diabetes was independent of

the administration of alloxan *per se*. It was not dependent on the sex or weight of the animal, nor upon the daily dosage of cholesterol, the form in which it was administered, nor the duration of cholesterol feeding. It was also independent of changes in body weight occurring during the course of our experiments and of the actual degree of hypercholesterolemia induced by the administration of cholesterol. In addition, there was no gross or histological evidence of a morphological basis for the inhibitory effect either in the aorta or in the other organs in which it was observed.

Only two factors were observed to be consistently associated with the inhibition of the expected morphological effects of cholesterol feeding, namely, the diabetic state and a degree of visible lipemia considerably greater than that observed in the control animals.

The inhibitory effect observed in these experiments would appear to depend upon some as yet undetermined factor or factors implicit in the diabetic state or closely associated with it. The experimental data presented demonstrate clearly that hypercholesterolemia is not the sole factor concerned in the genesis of experimental cholesterol atherosclerosis, but that another factor, or factors, rendered inoperative in our experiments must be essential to the production of the arterial lesions.

In view of the inhibitory effect on the development of experimental cholesterol atherosclerosis observed in alloxan-diabetic rabbits, the effect of alloxan diabetes on the retrogression of such arterial lesions was studied in another series of experiments. No effect on retrogression could be demonstrated within periods lasting up to a maximum of 4 months after the cessation of cholesterol feeding.

The results of our two series of experiments, considered together, indicate that the process of deposition of lipids in the arterial walls is governed by factors different from those that are operative in the process of removal of lipids after they have been deposited. The inhibition of the development of experimental cholesterol atherosclerosis in alloxan-diabetic rabbits must depend on interference with the process of deposition of lipids and not on a process of removal of lipids as fast as they are deposited.

Our experimental results find no direct application to the problem of arterial disease in human diabetes. Nevertheless, the experimental procedures that we have employed provide a new basis for the design of further experiments directed toward the elucidation of the nature of the unknown factors that govern the process of lipid deposition in the walls of arteries.

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be demonstrated by studying these two factors not only as they progressed following DCA administration, but also as they regressed following withdrawal of DCA.

One hundred and ten albino rats of the Sherman strain, averaging approximately 90 gm. in weight were divided into two groups. Forty-one animals served as untreated controls while the remaining 69 received DCA with 1 per cent saline as drinking water *ad libitum*. DCA was administered as one-third of a 75 mg. pellet (Schering cortate) subcutaneously implanted on the 1st day of the experiment and every 2 weeks thereafter. During the 4th week of the experiment renal function (7) and blood pressure (8) were determined in a group of 9 of the control and 18 of the DCA-saline-treated animals. Immediately following these procedures the control group and half of the treated group were sacrificed, their kidneys fixed, and then weighed. Pellets were removed from the remaining half of the DCA-saline-treated group which were then returned to tap water.

During the 6th week of the experiment renal function and blood pressure were again determined in a group of 8 control and 20 of the DCA-saline-treated animals, as well as in the group whose DCA-saline treatment had stopped 2 weeks earlier. Again, all animals were sacrificed except for half of the DCA-saline-treated group in which treatment was stopped at this time.

During the 8th week, the usual procedures were carried out in a control group, in a DCA-saline-treated group, and in the group whose treatment had been stopped in the previous period. Insufficient DCA-saline-treated animals remained to permit the experiment to be carried out beyond this period. Consequently, a subsidiary experiment was set up to determine the effects of stopping treatment in the 8th week. The base line data for control and DCA-saline-treated groups in the 8th week were again determined. Treatment was stopped in half of the treated group and their renal function and blood pressure determined again 1 week later.

Briefly, the clearance technique was as follows: PAH solution (12.5 mg./cc. in 2 per cent sodium sulfate) is injected into the lumbar region in accordance with a dose-body weight scale calculated to yield the desired plasma level of 5 to 7 mg. per cent at 50 minutes. Immediately following this injection, 3 cc. of warm 2 per cent inulin solution is injected intraperitoneally. The completion of this second injection marks the start of the urine collection period, and the rat is immediately placed into a metabolism funnel.

Fifty minutes after injection, the rat is picked up over the funnel and the bladder drained by suprapubic pressure, although micturition is usually free and spontaneous. Immediately following urine collection, 0.75 cc. of blood is obtained by heart puncture. Plasma and urine are then analyzed for inulin and PAH.

Blood pressure determinations are made with the tail plethysmograph using ether as anesthetic. While this method may fail to record a raised pressure in the occasional animal which is actually hypertensive, experience in many hundreds of animals indicates that it gives reliable and reproducible results. The actual pressure recorded is somewhat below the systolic level.

The results obtained in the two separate parts of the experiment (up to the 8th week, and then beyond this period) are presented in Table I. The number of animals indicated in each case refers to those sacrificed in that particular period. For simplicity, and in order to clarify the significant findings, the data have been rearranged graphically in Fig. 1. In this figure, the findings are presented as percentage deviation of the test group values from those obtained in a group of intact controls studied on the same day. Changes which are statistically significant ($p < 0.02$) are denoted.

TABLE I
*Blood Pressure, Renal Function, and Kidney Weight Determined in Groups of Sherman Rats
at Various Stages of Treatment with DCA and Saline, and Subsequent to the Cessation of
Treatment*

	Control	DCA-saline	DCA-saline treatment stop- ped in previous period
No. of animals.....	9	9	
Blood pressure.....	106 ±12	131 ±16	
C _{IN} , cc./100 cm. ²	0.34 ±0.03	0.34 ±0.03	
4th wk. CPAH, cc./100 cm. ²	2.48 ±0.33	2.70 ±0.45	
TmpAH, mg./100 cm. ²	0.125 ±0.014	0.121 ±0.021	
FF as per cent.....	13.7	12.9	
CPAH/TmpAH.....	19.8	22.2	
Kidney weight, mg./100 cm. ²	397 ±42	554 ±44	
No. of animals.....	8	10	9
Blood pressure.....	111 ±16	134 ±16	106 ±10
C _{IN} , cc./100 cm. ²	0.31 ±0.07	0.32 ±0.09	0.34 ±0.03
6th wk. CPAH, cc./100 cm. ²	2.47 ±0.27	2.81 ±0.76	2.73 ±0.37
TmpAH, mg./100 cm. ²	0.127 ±0.010	0.134 ±0.033	0.129 ±0.011
FF as per cent.....	12.5	11.4	12.4
CPAH/TmpAH.....	19.4	20.9	21.2
Kidney weight, mg./100 cm. ²	439 ±49	520 ±93	507 ±30
No. of animals.....	8	10	10
Blood pressure.....	107 ±13	151	97 ±17
C _{IN} , cc./100 cm. ²	0.33 ±0.03	Not graphed	0.39 ±0.03
8th wk. CPAH, cc./100 cm. ²	2.59 ±0.27		2.87 ±0.39
TmpAH, mg./100 cm. ²	0.120 ±0.017		0.131 ±0.015
FF as per cent.....	12.7		13.7
CPAH/TmpAH.....	21.7		21.9
Kidney weight, mg./100 cm. ²	459 ±45	602	501 ±39

ment either at 25, 37, or 51 days, blood pressure fell promptly to normal levels. Indeed, 6 days after cessation of DCA treatment which had been carried on for 51 days, a significant elevation of pressure was no longer demonstrable. Since blood pressure determinations were not carried out at times other than those specified in the table, the return of the elevated pressure to normotensive levels may have occurred even more rapidly than is here indicated.

Renal Function.—Renal function appeared undisturbed after 25 days and after 37 days of treatment with DCA-saline. This is in accord with our previous finding that Sherman animals do not develop renal functional changes as readily as do Wistar (9). Fifty-one days of treatment resulted in a significant increase in the glomerular filtration rate, and consequently, in the filtration fraction. Since in the two earlier periods renal function as here determined was unaffected by the DCA-saline treatment, it is not surprising that no real change was observed 12 days after cessation of treatment in either case. The marked deviation in filtration rate observed after 51 days of treatment disappeared 6 days after treatment was stopped.

The data concerning renal function fall in the same direction as others which we have reported and would, alone, suggest that the elevation in blood pressure is independent of renal functional derangement. Further, since change in renal function may only be observed relatively late in the course of treatment, the idea that it results from the elevated pressure might well be entertained. This suggestion is, however, contradicted by the observations concerning renal weight.

Kidney Weight.—Bearing in mind the obvious fact that changes in renal mass can probably not occur as rapidly as alterations in blood pressure, a remarkable parallel between these functions was observed. Elevation of the blood pressure even at the earliest date studied was accompanied by an increase in kidney weight, while restoration of the blood pressure was accompanied by a return towards normal of kidney weight. Since renal function was maintained only at the normal level despite this increase in size, it seems reasonable to assume that this process is a compensatory hypertrophy.

It is of some interest in relation to the theory of renal function tests that this renal involvement becomes apparent at once when renal function is related to actual renal mass.

The apparent correlation between renal mass and blood pressure was subjected to statistical analysis. In Fig. 2, renal mass in the DCA-saline-treated animals is plotted as a frequency distribution against blood pressure, the data being taken from part one of the experiment. The regression line for the data in which b , the coefficient of regression is 0.124, is statistically significant. Since this graph represents all treated animals, whether or not pellets were removed, the significant regression assumes even greater meaning. The

conclusion that the kidneys are in some way involved in DCA hypertension from the beginning of the process appears unavoidable.

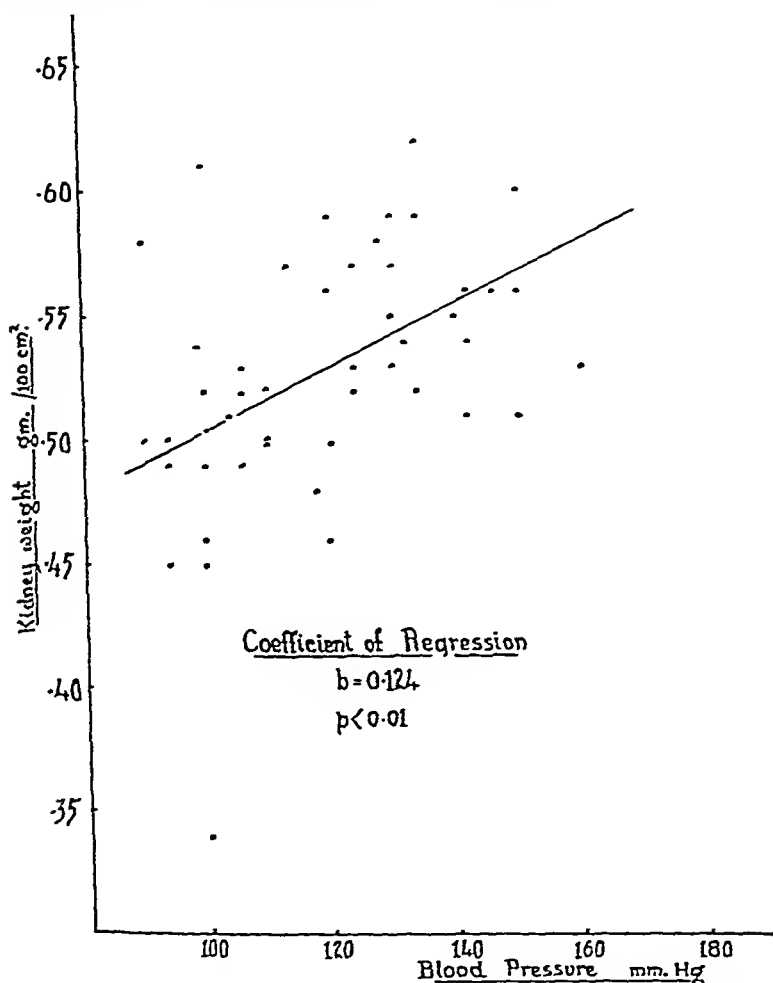


FIG. 2. Renal mass in DCA-saline-treated animals plotted as a frequency distribution against blood pressure.

Experiment 2

Since the kidneys are thus apparently immediately involved following DCA administration, two major possibilities concerning the mechanism suggest themselves—(a) the kidneys liberate a pressor substance upon stimulation by DCA or (b) the kidneys are actively concerned in the excretion and possible inactivation of DCA.

It seemed to us that the first step in distinguishing between these possibilities would be to examine the effects of DCA on blood pressure in the absence of the kidneys. The basic principle followed was to administer DCA until the blood pressure had attained a predetermined level, and then to nephrectomize the animals. A fall in pressure following nephrectomy would offer strong support to the idea that the pressor effect of DCA had been mediated by the kidney, perhaps through stimulation of a renal pressor mechanism. On the other hand, a rise in the blood pressure following nephrectomy would indicate that the pressor action of DCA was a more direct phenomenon.

Four separate experiments were carried out. In two of these, the blood pressure was elevated to a significant but low degree before nephrectomy so that either a fall or a rise might be easily discerned. Two were performed at an earlier stage in DCA treatment when the blood pressure was not yet significantly elevated. Since all four experiments yielded the same fundamental result only one experiment of each group is here reported.

Experiment 2a.—Twenty-eight male albino rats of an inbred Wistar strain and approximately 150 gm. in weight were maintained for 29 days. Eight animals served as untreated controls while the remaining 20 received a DCA pellet (one-third of a 75 mg. cortate pellet) as a subcutaneous implant on the 1st and 14th days of the experiment. On the 20th day, the left kidney was removed from each of 12 of the DCA-treated animals and on the 27th day of the experiment the remaining kidneys were removed. Blood pressure was determined at 1 or 2 day intervals beginning on the 18th day. The findings for this experiment are presented in Fig. 3. Seven of the 12 nephrectomized animals survived for the blood pressure determination 24 hours after complete nephrectomy, 2 for the 48 hour period.

Blood pressure was significantly elevated in the DCA-treated groups at the time of the first blood pressure determination on the 18th day. A fall in blood pressure occurred immediately following removal of one kidney but this was only temporary. In contrast, 24 hours after removal of the second kidney, a significant elevation above both untreated and DCA-treated controls was observed in the blood pressure of the nephrectomized animals. This result is the more remarkable since it occurred despite the undoubted operative shock, a factor not present in the control groups, and was observed not only as a group average but also in 6 of the 7 survivors, while the pressure of the seventh animal did not fall. The further elevation observed at 48 hours cannot be considered since it is based on only two survivors.

Experiment 2b.—Thirty male albino rats of an inbred Wistar strain, approximately 150 gm. in weight, were maintained for 14 days. Fifteen of these animals received 2 pellets (one-third of a cortate pellet) on the 1st day of the experiment and a third pellet on the 6th day. On the 12th day, 9 control and 9 DCA-treated animals were subjected to a one stage bilateral nephrectomy. Blood pressure was determined daily beginning on the 11th day. The results are presented in Fig. 4. All animals survived in good shape for 24 hours, but only 4 of the DCA-treated and 1 of the untreated nephrectomized animals were available for the 48 hour determination.

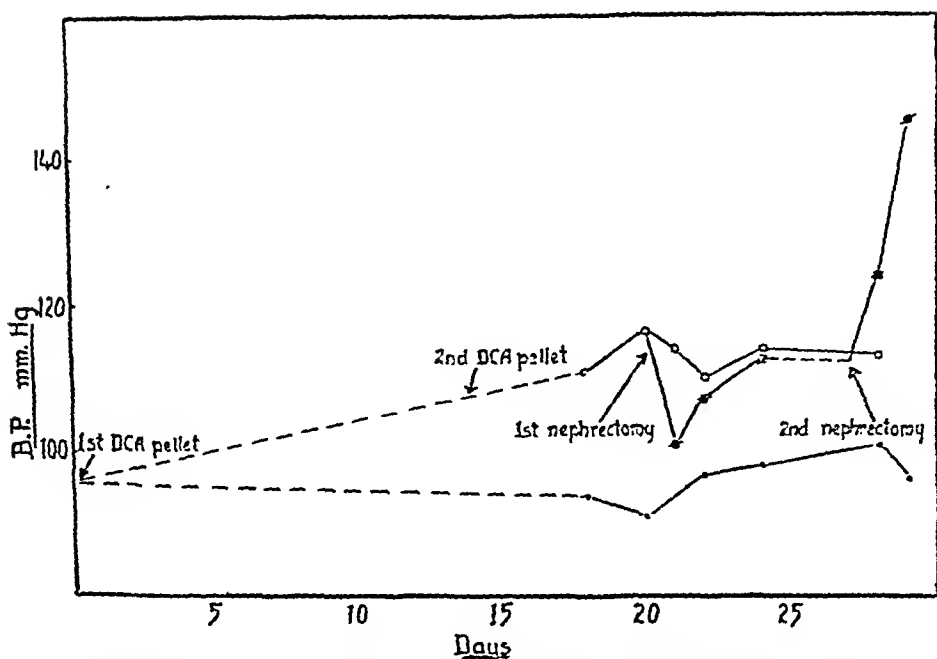


FIG. 3. The blood pressure in DCA-treated rats before and after removal of the kidneys.

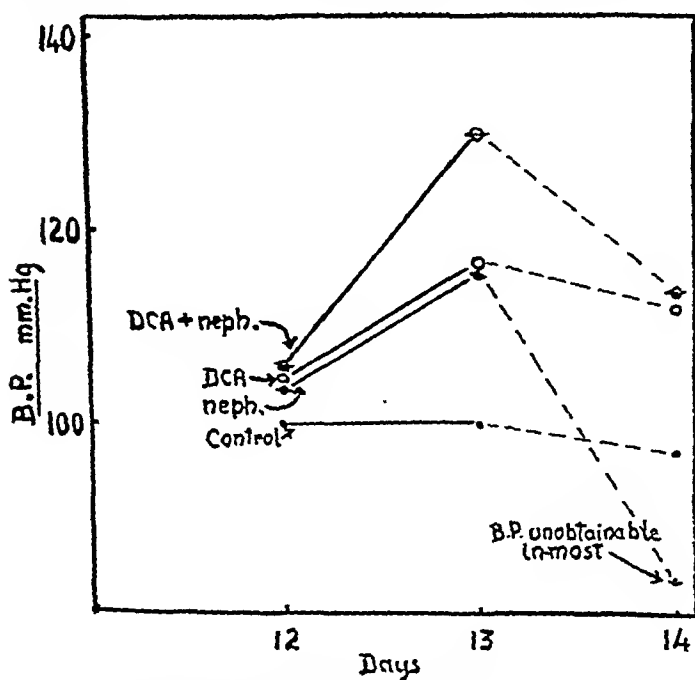


FIG. 4. The blood pressure in DCA-treated rats before and after removal of the kidneys.

Prior to nephrectomy no elevation in blood pressure was observed in any group although 2 days later the DCA-treated animals were beginning to show a rise. Twenty-four hours after removal of both kidneys blood pressure was elevated in both DCA-treated and untreated nephrectomized animals. In the case of the untreated group, this finding was significant but resulted from an elevation of pressure in only 3 of the 9 animals. On the other hand, all 9 DCA-treated nephrectomized animals participated in the observed elevation in this group, an elevation statistically significant not only in reference to the untreated control level but also in reference to the moderate elevation in the two other groups. The 48 hour findings are based on too few data to permit discussion.

DISCUSSION

Earlier workers had observed that when DCA was administered to suitably sensitized animals, hypertension and renal damage occurred. They assumed that the kidneys were primarily involved in DCA hypertension, although such a conclusion was not necessarily warranted by the data. Reinvestigating the problem from a functional approach we drew attention to the absence of renal functional change at a time when hypertension following DCA administration was well established, and pointed to the similarity of this observation to the findings in essential hypertension. Further exploration aimed at both the progression of DCA hypertension and its regression upon cessation of treatment showed, however, that the kidneys are involved from the start in the process. Attention is drawn particularly to those data which show clearly how compensatory hypertrophy may completely mask the presence of interference with renal function.

After removal of both kidneys, an aggravation of the hypertension was observed in DCA-treated animals. It is thus unlikely that the kidney enlargement reflects a stimulated production of renal pressor material, but it seems reasonable to suggest that the kidney is actively concerned with the excretion and possible inactivation of the steroid.

It is also possible to explain these findings according to Grollman's view that the kidney normally liberates an antihypertensive factor (10). In the present state of information, however, it is not possible to distinguish between the idea of a renal antihypertensive factor on the one hand and the renal destruction of a pressor agent on the other.

SUMMARY

Desoxycorticosterone acetate in pellet form was administered for 51 days to albino rats of the Sherman strain which also received 1 per cent saline as drinking water. Treatment was stopped in representative groups at 25, 37, and 51 days so that the regression of blood pressure and renal changes could be ob-

served. It was noted that both the elevation in blood pressure during treatment and its reversal when treatment was stopped were closely correlated with corresponding changes in renal mass. In the time for which the process was studied it did not become irreversible.

Removal of both kidneys from DCA-treated animals aggravated the hypertension, suggesting that the kidneys are actively concerned with the excretion and possible inactivation of the steroid.

The authors wish to thank Dr. E. Henderson and Dr. W. Stoner and Mr. W. E. Fielding of the Schering Corporation for the cortate pellets and Dr. W. Boger of Sharp and Dohme Inc., for the sodium *p*-aminohippurate used in this work.

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EFFECTS OF PROTEINURIA ON THE KIDNEY*

PROTEINURIA, RENAL ENLARGEMENT, AND RENAL INJURY CONSEQUENT ON PROTRACTED PARENTERAL ADMINISTRATION OF PROTEIN SOLUTIONS IN RATS

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PLATES 32 TO 34

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Renal damage due to cast formation and tubular plugging by protein material, is well known in diseases associated with hemoglobinuria (1), Bence-Jones proteinuria (2), and "albuminuria" (3). The magnitude of this type of damage in Bright's disease is greatly influenced by the globulin content of the urine (4). Whether proteinuria results in renal damage other than that caused by the blockage of tubules, has not been fully determined. Cast formation may sometimes be a result of damage, as well as a cause of damage (5), and tubular lesions which did not appear to be due to obstruction have been observed with hemoglobinuria (6). However, as emphasized in Lucké's review (7), hemoglobinuria as observed clinically is often associated with shock, dehydration, and other factors, which may themselves contribute to the production of renal injury, and in diseases characterized by "albuminuria" or Bence-Jones proteinuria, one cannot always distinguish between possible effects of protein on the kidney, and other manifestations of the disease in question. The use of hemoglobin in investigations designed to determine the effects of protein itself on the kidney, is complicated by the presence of the prosthetic group.

With the advent of the treatment of nephrosis by the parenteral administration of large amounts of protein, usually with an exaggeration of the degree of proteinuria during the period of therapy, the question of the possible harmful effects of proteinuria on the kidney assumed greater importance. The same question also enters into the choice of diet in renal diseases, since the urinary protein often is increased by raising the dietary protein level.

In order to study the effects of proteinuria on the kidney, and particularly to determine whether the passage of protein through the kidney might result in renal damage other than that attributable to tubular obstruction, it was decided to attempt the production in rats of continuous proteinuria of abnormal degree, for extended as well as brief periods of time, and to follow the changes which occurred in renal morphology and composition during and following cessation of treatment, together with the changes in blood, urine, and other organs.

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Preliminary experiments indicated that fairly continuous additions of various proteins to the blood stream, and increases in the amount of protein in the urine could be induced by twice daily intraperitoneal injections of solutions of the proteins. Efforts were made to adjust fluid and electrolyte intake, and the amount of protein administered, in such a manner that tubular obstruction would not occur in the majority of animals, and that the results would not be influenced by differences in urine volume, dehydration, electrolyte imbalance, or shock.

In addition to observing the effects of the protein injections on the kidney, some information was obtained concerning the degree and duration of retention of the various proteins in the plasma, and it was possible to make observations of a preliminary nature bearing on the questions of the mechanism of proteinuria, the passage of protein through the glomerular membranes, and the reabsorption of protein by the tubules.

EXPERIMENTAL

Materials and Methods

Animals.—Two hundred and eighty-five male albino rats of a Sherman strain were given injections as described below. The animals were approximately 24 days old when the injections were started. The maximum variation in initial weight of animals used in any series was less than 10 gm. Approximately 100 large adult rats were used as donors for rat serum which was administered to some of the experimental rats, and about 50 additional rats were employed in auxiliary experiments.

Protein Solutions.—Three per cent solutions of the following proteins, each dissolved in 0.45 per cent sodium chloride solution, were employed for injections in some of the treated groups of animals: gelatin,¹ human albumin,² and bovine gamma globulin.³ Phenyl mercuric borate 0.002 per cent was present in the gelatin solution and was added to the other solutions. In addition to the experiments employing 3 per cent protein solutions, a few rats received a solution containing approximately 6 per cent gelatin in a physiological solution of sodium chloride, with 0.004 per cent phenyl mercuric borate.¹ The solutions were prepared and handled aseptically, and refrigerated when not in use.

Rat Serum.—Rat serum diluted with an equal volume of distilled water (giving approximately a 3 per cent protein solution in half-physiological salt solution), and with 0.002 per cent phenyl mercuric borate added, was used for injections in two groups of animals. The serum administered to the first group was obtained from adult rats decapitated after ether anesthesia of sufficient depth to cause respiratory depression or paralysis. When it appeared that certain undesirable effects of the serum might be due to ether retained by the serum, the serum used for

¹ Purified gelatin in isotonic solution of sodium chloride, oncotic pressure 70 mm. Hg, supplied by the Upjohn Company, Kalamazoo, Michigan, diluted with an equal volume of water. The stock solution containing approximately 6 per cent gelatin was administered without dilution to a few animals of series 9.

² Normal serum albumin (human) concentrated, salt-poor, E. R. Squibb and Sons, supplied by the American Red Cross.

³ Fraction II from bovine plasma, supplied by The Armour Laboratories, Armour and Company, Chicago.

the second group was obtained after decapitation, from large rats which had been placed in a nitrogen chamber until respiration stopped. These animals frequently remained in an atmosphere with little oxygen for 5 minutes or longer, and convulsions during this period were not uncommon. In both cases, the blood from the donor animals was collected with minimal contamination into tubes by means of paraffin-covered funnels and allowed to clot. The serum was separated promptly by centrifugation, placed in tubes, and immediately frozen. Portions of serum sufficient to last 24 to 36 hours were thawed and diluted as needed for injections, with constant refrigeration of the diluted serum.

Control Solutions.—One per cent urea, and 3 per cent casein hydrolysate,⁴ each dissolved in 0.45 per cent sodium chloride solution, and the 0.45 per cent sodium chloride solution alone, were administered individually to control groups of animals. Phenyl mercuric borate 0.002 per cent was added as a preservative. The solutions were aseptically prepared and handled, and refrigerated between injections. In some of the later experiments, distilled water rather than half-physiological salt solution was used in the preparation of the urea and protein hydrolysate solutions in order to decrease peritoneal irritation.

General Plan of Experiments.—Comparable groups of the young rats were given, for various periods of time, twice daily intraperitoneal injections, 1 cc. per 10 gm. of body weight at each injection, of the protein, serum, and control solutions. The different series of injections, and the determinations which were done on the animals of each series are summarized in Table I. The injections were made without anesthesia, through a 21 gauge needle, care being taken to maintain aseptic conditions and to avoid injury of the liver and intestines. In order to insure an equal fluid intake and the elimination of urine of comparable volume and concentration by the animals of the different groups, *no fluid* except that administered in the injections already described was allowed. A stock diet⁵ was offered *ad libitum*. For several days prior to and during the urine collections, a low residue synthetic diet⁶ was substituted for the stock diet in order to decrease the bulk of the feces. Because vitamin-free injected materials were to some extent utilized in place of food by animals of some of the groups, complete vitamin supplements⁷ were offered *ad libitum* to all animals. A complete salt mixture⁸ was also supplied separately so that the animals could adjust their own salt intake to some extent. The animals were weighed at intervals of a few days and the amounts of injected materials were increased as indicated. Urine was collected from time to time for protein determinations. Animals from various groups were autopsied at intervals during, at the end of, and at periods following terminations of injection periods. Blood was collected for study, weight of kidneys was determined, and kidney tissue was preserved for chemical and histological study. Animals not autopsied during, or at the end of, injection periods were given water *ad libitum* beginning 24 hours after the final injection. The occasional animals which became seriously ill or died were not included in the determinations of kidney size.

Urine Collections.—Urine was collected by means of metabolic cages placed above large paraffin-coated funnels which drained into flasks containing a little toluene. Because of the

⁴ Amino acids—I. C., lyophilized (acid hydrolysate of casein with added tryptophane) supplied by Biochemical Division, Interchemical Corporation, Union, New Jersey.

⁵ Purina fox chow.

⁶ The diet employed was the same as that described elsewhere (8), with 0.3 per cent choline chloride added. The changes in the diet did not appear to influence appreciably the kidney weight-body weight ratios or the amount of protein in the urine. The dietary treatment of protein-treated and control groups was the same in each experiment.

⁷ The vitamin mixture was the same as that used in the synthetic diet (8), with added choline chloride.

⁸ Osborne-Mendel salt mixture No. 2, Eimer and Amend, New York.

small size of the animals, from 3 to 6 rats were placed together in each cage. Collections were made for periods of from 12 to 24 hours. When injections were continued during the periods of urine collections the animals were not returned to the metabolic cages until leakage from peritoneal cavity, if present, had ceased. Since fairly uniform results were obtained, it was thought that subsequent leakage did not occur. Because of technical difficulties, the collections were only approximately quantitative. As indicated in Table I, urine collections were made at various times after termination of injections, as well as near the end and sometimes at earlier stages of the injections.

Urine Examinations.—The urine specimens were cleared by centrifugation, and filtered if necessary. Volume was recorded, and protein concentration determined by the Shevky-Stafford (9) or the biuret (10) method. Gelatin was precipitated by addition of tungstate or Tsuchiya's reagent after removal and estimation of other proteins by addition of an equal volume of 10 per cent trichloroacetic acid. Human albumin was estimated in a few cases by determination of the turbidity produced by addition of antiserum prepared from rabbits.⁹ Bovine globulin was tested for qualitatively by serological precipitation, and the relative globulin content of urines from various groups was estimated by comparing the precipitates formed by adding equal volumes of 44 per cent sodium sulfate, after adjusting pH of the urine specimens to 7.4 (11).

Urine sediment was examined microscopically in a few instances.

Blood Examinations.—At the time of autopsy, during, or sometime after termination of the injections, blood was collected into heparin-containing flasks after decapitation of the animal. Total plasma protein and hemoglobin levels were estimated from specific gravity determinations by the copper sulfate method (12). Urea was determined by the hypobromite method (13). Total plasma nitrogen and plasma albumin determinations were done in a few instances by a micro Kjeldahl method (14), using pooled plasma from the various groups.

Examination of Organs.—After comparable animals of different groups had been weighed, killed by decapitation, and thoroughly drained of blood, the peritoneal cavities were inspected for free fluid, and for evidence of peritoneal irritation, infection, or injury to organs. When fluid was present in the peritoneal cavity, the weight of the animal was corrected by deducting the weight of the free fluid. Tissues were observed for evidence of dehydration or overhydration. The size, color, and other gross features of kidneys, liver, and spleen were specifically noted. Differences in size and shade of kidneys were most impressive when the organs from comparable control and protein-injected animals were compared directly by placing the kidneys side by side. The kidneys and sometimes the liver and spleen were removed for weighing, and tissue was preserved for histological study of gross and microscopic sections, and for total nitrogen and moisture determinations.

Determination of Relative Weight of Kidneys.—The animals of the different groups were of approximately the same weight at the beginning of the injection periods, and the growth rate of all the animals was fairly uniform, at least for several weeks. In experiments which were continued for a number of weeks, there was sometimes considerable variation in weight of the animals at the time of autopsy. In the early experiments, the kidneys were removed without attached tissue, but with capsules in place, and were weighed accurately. After it was observed that some of the injected solutions produced significant thickening and increase in weight of the renal capsules, the capsules of all kidneys were removed carefully before weighing. In order that the kidneys of various animals might be compared more accurately, the ratio of kidney weight to corrected body weight was determined in each case. In each series of experiments, animals of various groups were treated similarly. In most cases, autopsies were

⁹ We are indebted to Dr. Henry G. Kunkel for the determinations of human albumin in urine and renal cortical tissue.

performed about 18 hours after the last injection, with no oral fluid during the intervening period. In some of the early experiments, however, the time interval between the last injection and autopsy was less than 18 hours.

Histological Examinations of Kidneys and Other Organs.—After the kidneys were weighed, they were sectioned along the median sagittal plane, and direct comparisons made of kidneys of animals of various groups. Kidney tissue was then fixed in alcohol-formol-acetic acid,¹⁰ or sometimes in 10 per cent neutral formalin or in absolute alcohol. Paraffin sections were prepared as routine, and stained for microscopic examination with hematoxylin and eosin or with Giemsa's stain, and frequently with Mallory's connective tissue stain. A few sections from kidneys of gelatin-treated animals were fixed in absolute alcohol and stained with eosin and with several other stains in absolute alcohol, without passage through aqueous solutions. Frozen sections of formalin-fixed tissue were stained for fat in a few instances. Liver sections from some animals and sections of other organs from occasional animals also were studied microscopically.

Determinations of Water and Other Constituents of Cortical Tissue.—Cortical tissue was prepared by bisecting decapsulated kidneys and removing the medullary portion. The cortical tissue was then pressed against blotting paper to remove free fluid present in tubules and blood vessels.

Moisture determinations were made on kidneys from most of the animals. This was done by placing the cortical tissue from one-fourth to one-half of a kidney in a weighing bottle, accurately determining the wet weight of the tissue, drying in an oven at 104°C. for 6 hours, and again weighing. The percentage loss of weight by the wet tissue on drying was then calculated.

After tissue had been taken for sections and for moisture determinations, the remaining cortical tissue was placed in tubes, weighed, and frozen. Total nitrogen determinations were performed on this tissue in a few cases by a Kjeldahl method (14). In some other cases, the tissue was ground in water or saline with sand or by means of a Potter homogenizer. The solutions were cleared by high speed centrifugation, and gelatin, human albumin, and bovine globulin were precipitated as already described for the proteins in urine. Results on kidney tissue from control and protein-treated animals were compared in each instance. In a few cases, the protein injections were discontinued and the animals given water for 2 to 7 days before autopsy in order to eliminate most of the injected protein from blood and urine, small amounts of which were retained by the renal tissue. Although a few quantitative estimations of the specific protein were made, the results were of only qualitative significance because certain factors were not well controlled.

RESULTS

Most of the animals which received injections of the various substances remained well and grew fairly normally. The animals which received injections for the longer periods grew from weanlings to rats weighing approximately 200 gm. without receiving any fluids by mouth. Disturbances in health and growth of animals due to intraperitoneal infections or injuries produced at the time of injections, were rarely encountered. There was no evidence of sensitization of the protein-injected animals to the proteins, though temperatures of the injected animals were not determined following injections and sera were not examined for antibodies against the proteins. The impression was obtained that the animals which were given gelatin were less excitable and struggled less

¹⁰ Eighty per cent alcohol 900 cc., 40 per cent formalin 50 cc., and glacial acetic acid 50 cc.

during injections than those of other groups. In the early experiments, the animals receiving protein hydrolysate appeared to be less able than animals of other groups to tolerate periods of more than 12 hours without an injection of fluid. This difference was partially removed by administering the protein hydrolysate in water rather than in sodium chloride solution, and by offering a complete salt mixture separately.

Data obtained from groups of animals injected with rat serum are included in tables and charts along with data from other groups. However, since the experiments with the homologous serum were not considered entirely satisfactory, they will be discussed separately.

Absorption of Injected Materials from Peritoneal Cavity.—The control (non-protein) solutions were absorbed somewhat more rapidly from the peritoneal cavity than were the protein solutions. In most experiments, even the protein solutions were fairly completely absorbed during the period from one injection to the next, and only in the case of one group receiving the usual 3 per cent solution of gelatin (series 4), the group receiving 6 per cent gelatin (series 9), and particularly the groups receiving rat serum did intraperitoneal accumulations of injected material present a serious problem. The animals receiving urea became dehydrated and the peritoneal surfaces did not have the normal moist appearance, though the hemoglobin levels did not appear to rise appreciably.

Changes in Plasma Proteins and Hemoglobin Levels Resulting from Injections.—Plasma protein and hemoglobin levels, which were determined in animals of the various groups at the time of autopsy of the animals, during the course of, and at intervals following the termination of injection periods, varied somewhat from group to group, and with the length of the injection period, and with the period of time elapsing between the last injection and the autopsy of the animal, but, qualitatively, the results were similar. Data obtained from animals of series 3, 6, and 7, are presented respectively in Tables II, III, and IV. Little or no change in serum protein or hemoglobin levels was observed with gelatin injections. Unfortunately studies were not done on animals of series 4 receiving the usual gelatin solution, or on those of series 9 receiving the more concentrated gelatin solution, some of which retained fluid in the peritoneal cavity, and some of which developed severe tubular damage. Albumin produced significant elevations in total plasma protein, plasma albumin, and in the ratio of albumin to globulin, with a coincident decrease in the hemoglobin level. Globulin induced an even greater rise in total plasma protein and plasma globulin, but the decrease in hemoglobin concentration was of about the same magnitude as with albumin. After termination of injections, the plasma protein and hemoglobin values returned fairly promptly toward normal.

Effect of Injections on Blood Urea Levels.—The animals receiving injections of control and protein solutions, including those of the long term experiments were found at the time of autopsy to have blood urea nitrogen values ranging between 12 and 28 mg. per cent, with the majority in the lower two-thirds of the

range, and there was no consistent difference between control and protein-treated animals. Some of the higher values were found in animals which appeared ill, but neither illness nor high urea nitrogen values were more common in protein-treated than in control animals, and neither could be attributed with any degree of certainty to specific effects of the injected materials on the kidneys. In animals given continued injections for prolonged periods and then observed for several weeks or months on a normal regime of food and water by mouth, the blood urea nitrogen values were still found to be normal. Blood urea levels were not determined in series 4 and 9 where retention of fluid in the peritoneal cavity, and renal damage, were observed in some of the animals receiving gelatin.

TABLE II
Total Plasma Protein and Hemoglobin Levels of Rats of Series 3

Injected solutions*	Total plasma protein	Hemoglobin
	<i>gm. per cent</i>	<i>gm. per cent</i>
Saline	6.5	10.6
Urea	6.2	9.9
Casein hydrolysate	6.5	11.0
Gelatin	6.4	10.1
Albumin	8.5	8.4
Globulin	9.5	8.3

The animals were autopsied about 6 hours after the last injection, following an injection period of 2 weeks. Each figure represents an average from specific gravity determinations on 3 rats by the CuSO_4 method.

* Solutions previously described in text.

Influence of Injections on Proteinuria and Urine Output.—Rapidly occurring diuresis after each injection was noted in animals receiving injections of urea, and the volume of urine excreted in 24 hour periods by these animals was usually somewhat greater than that excreted by comparable animals of other groups, despite the fact that the fluid intakes of comparable animals of all groups were equal. The rate of urine excretion was more uniform throughout the 24 hour periods in the protein-treated animals than in those receiving saline and amino acids, but the total urine volumes of animals of all these groups were approximately equal.

Urine from saline-injected animals always contained small amounts of protein, nearly always less than 1 gm. per liter, and often in the range of 0.5 gm. per liter or less. The total protein excreted per day varied with the size and age of the rat, but after an injection period of 2 weeks, starting with weanling rats, the amount was usually found to be between 1 and 2 mg. per rat per 24 hours. The amounts of protein excreted by the animals receiving amino acids

TABLE III
Plasma Protein and Hemoglobin Levels of Rats of Series 6

Injected solutions*	18 hrs. after final injection			2 days after final injection		5 days after final injection		10 days after final injection		
	Plasma protein†	(Plasma protein) A/G‡	Hb‡	Plasma protein†	Hb‡	Plasma protein†	Hb‡	Plasma protein†	(Plasma protein) A/G‡	Hb‡
	gm. per cent		gm. per cent	gm. per cent	gm. per cent	gm. per cent	gm. per cent	gm. per cent		gm. per cent
Saline	5.0	(6.0) 1.8	12.2	5.5	12.7	5.7	12.6	5.7	(5.7) 1.6	11.4
Casein hydrolysate	5.2	—	13.3	—	—	—	—	—	—	—
Gelatin	5.3	(4.8) 1.7	12.1	—	—	5.7	12.7	5.4	(5.5) 1.6	10.8
Albumin	6.5	(6.7) 3.0	10.2	5.8	11.7	—	—	5.4	(5.3) 1.6	10.9
Globulin	8.6	(9.5) 0.33	10.5	8.0	13.0	6.5	14.0	6.0	(5.9) 1.3	11.2

After an injection period of 9 days, the animals were autopsied at intervals as indicated in the table. Animals were given water *ad libitum* beginning 24 hours after the final injection.

* Solutions previously described in text.

† Average values calculated from specific gravity determinations on a total of 40 rats by the CuSO_4 method.

‡ Single determinations on pooled plasma, using Howe separation and micro Kjeldahl.

TABLE IV
Total Plasma Protein and Hemoglobin Levels in Rats of Series 7

Injected solution*	Total plasma protein	Hemoglobin
	gm. per cent	gm. per cent
Saline	5.7	12.5
Urea	5.6	12.8
Casein hydrolysate	6.0	11.9
Rat serum	7.8†	11.4

Animals were autopsied about 18 hours after the last injection, following an injection period of 9 days. Each figure represents an average value calculated from specific gravity determinations on 6 rats by the CuSO_4 method.

* Solutions previously described in text.

† A single determination on pooled plasma indicated that the albumin fraction was only slightly elevated.

and urea were in the same range as that of the saline animals, though the values for the urea-injected animals appeared to be consistently slightly higher than those of the other control groups, perhaps due to the diuretic effect. Additions

of sodium sulfate according to the method for globulin precipitation, to urines from the control groups, produced not more than a faint cloud, and most of the protein was presumably albumin. Urine specimens collected 1 to 2 weeks after starting injections, from the protein-treated animals, almost always contained more than 1 gm. of protein per liter of urine. The animals receiving albumin sometimes excreted as much as 5 gm. per liter or more over the 24 hour period. Globulin induced less marked elevations in urinary protein levels, while gelatin injections were usually accompanied by greater degrees of proteinuria than those observed with albumin.

The increase in urinary protein in animals injected with gelatin was due principally to the presence in the urine of gelatin; the other urinary proteins (precipitated by an equal volume of 10 per cent trichloroacetic acid) did not appear to be regularly or markedly altered. Gelatin appeared in the urine and was occasionally seen in the glomerular capsules before there were appreciable changes in kidney size or visible alterations in the tubular cells. In animals injected with human albumin, preliminary determinations of the human albumin in the urine by the serological precipitin reaction suggested that only a part, probably less than one-half of the urinary protein increment was composed of the injected human albumin. The remainder was presumably rat protein, probably chiefly albumin. As in the case of the albumin, it appeared unlikely that the injected globulin was present in the urine in sufficient quantities to account for all the increase in urinary protein which accompanied injections of that protein.

In animals receiving injections of albumin and globulin, the urinary protein levels, like the plasma protein levels already discussed, remained definitely elevated for at least several days after termination of the injections. However, when urine specimens from the various groups receiving proteins were examined after periods of 2 weeks to 2 months, the urinary protein levels were found to be within the same range as in the specimens from comparable control animals.

Examination of urinary sediment from protein-treated animals revealed no abnormalities.

Gross Changes in Organs Resulting from Injections.—Examinations of the peritoneal cavities sometimes revealed adhesions between liver or intestines and omentum or adjacent organs, due to puncture wounds produced at the time of injections. Significant peritoneal infections were never recognized, if present. Prolonged injections of urea solution, and particularly of protein hydrolysate solution, appeared to produce peritoneal irritation, with thickening and opacity of peritoneal surfaces including hepatic and renal capsules. In the case of urea, the peritoneal surfaces were often less moist than with the other solutions. Saline and the 3 per cent protein solutions produced no peritoneal changes. However, the more concentrated gelatin solution used in series 9 produced definite peritoneal thickening, with rounding of the liver edges.

Occasional animals, without respect to treatment, were observed to have large dark spleens.

It was noted during the early periods of the study that the kidneys of animals which had received repeated injections of gelatin appeared enlarged and pale. The correctness of this observation was confirmed by side by side comparisons of the kidneys of control and gelatin-treated animals (Figs. 1 and 3). When sagittal sections of kidneys from gelatin-injected animals were compared with similar sections from comparable control kidneys, it was further evident that the enlargement of the kidneys from animals receiving gelatin was principally in the cortical portion. The cortical tissue appeared pale particularly through the outer two-thirds of its depth, and was separated from the pale medullary tissue by a narrow dark band at the corticomedullary junction.

In experiments employing albumin and globulin, similar but usually somewhat less marked renal enlargement was noted (Figs. 2 and 3). Paleness of some degree was seen frequently with injections of these proteins, particularly when enlargement of considerable degree occurred, but it was usually much less than with gelatin.

No gross alterations, other than the differences in size and depth of color, were noted in any of the kidneys, and the pelves and ureters always appeared normal.

There appeared to be no changes in the liver or spleen attributable to injections of protein, though these organs were accurately weighed in only a few cases. No changes were noted in the adrenal glands, which were not studied extensively.

Change in Relative Size of Kidneys Due to Injected Substances.—Since there was always some variation in size of the experimental animals at the time of autopsy, the feasibility of expressing the relative kidney size as the ratio of the weight of both kidneys divided by the weight of the animal, was investigated. It was found that this ratio was quite constant over the range of variation in animal size encountered in the individual experiments, particularly in those of not more than a few weeks' duration. This ratio, multiplied by 100, decreased steadily with growth of the animals, from approximately 1.25 in the youngest rats autopsied to about 0.67 in the rats of series 1 which were autopsied after being observed for 2 months at the end of the 6 weeks injection period.

In the early experiments, the kidneys were weighed without decapsulation. However, after the introduction of urea, amino acid, and rat serum solutions, it was observed that these substances caused sufficient thickening and increase in weight of the renal capsules to alter appreciably the kidney weight-body weight ratios, and in subsequent studies the kidneys were carefully decapsulated before weighing.

The animals receiving urea and protein hydrolysate solutions, like those receiving saline alone, exhibited little change in relative kidney size. The kidney weight-body weight ratios of the animals receiving urea were usually slightly

elevated compared with those of the other control groups, perhaps because of a decrease in body weight due to dehydration.

Protein solutions uniformly caused an increase in relative kidney size and weight. The most rapid enlargement appeared to occur in the early part of

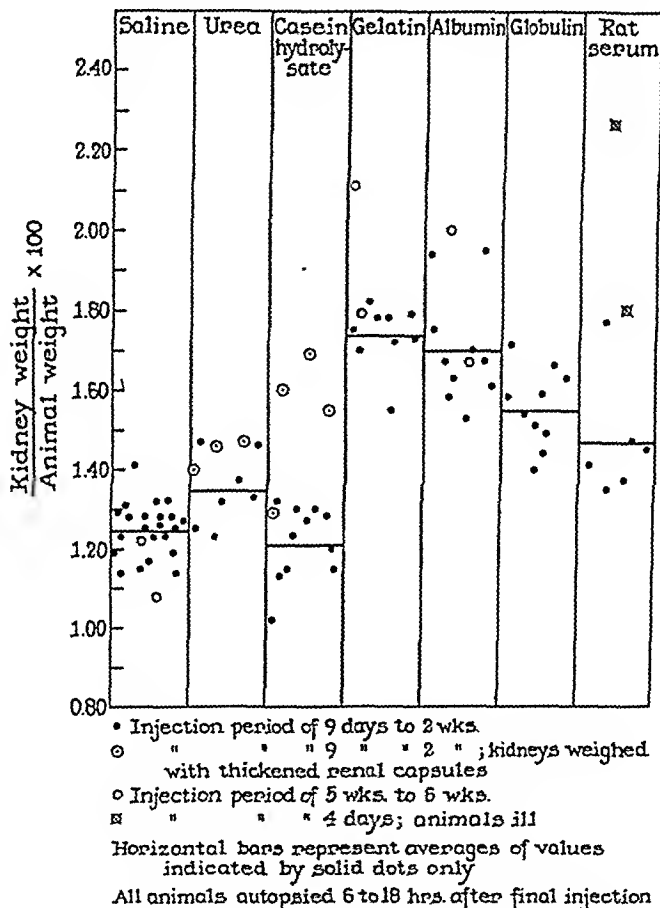


CHART 1. Relative renal size of comparable rats injected with various protein and control solutions.

the injection periods, but the enlargement was maintained as long as the injections were continued.

Gelatin caused the most rapid and usually the most marked increase in kidney size. Definite enlargement occurred within a few days, and perhaps within 1 day. Albumin ultimately caused almost as great enlargement as gelatin but the enlargement appeared to develop more slowly. Globulin produced less enlargement than gelatin or albumin.

Chart 1 shows the results of determinations of relative renal size which were made in the animals receiving injections for 9 days or longer and autopsied within 1 day after the final injection. The animals were from series 1, 2, 3, 6, and 7, with the two ill animals from series 8. Chart 2 contains further data from series 4 on the enlargement produced by short term injections of gelatin.

Reversibility of Renal Enlargement after Termination of Injections.—Chart 2 shows the changes in relative kidney size over a period of 8 days in rats of series 4, given 3 single injections of gelatin solution, compared with those of rats given injections of saline and casein hydrolysate. For some unknown reason,

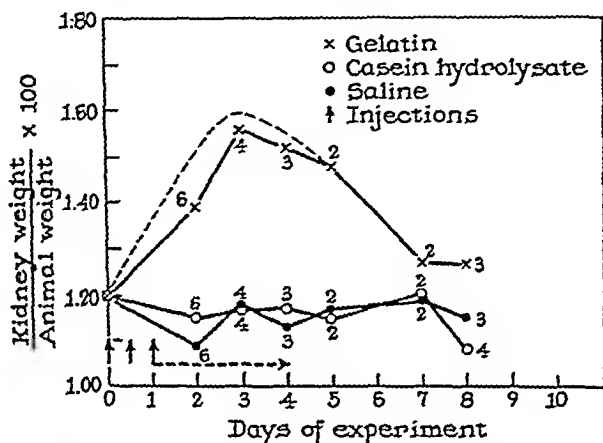


CHART 2. Showing changes in relative renal size on successive days produced by three injections of gelatin, compared with control injections. Absorption of gelatin was unusually slow, as indicated by horizontal arrow, and weight of animals was not corrected for weight of peritoneal fluid. Broken line indicates approximate correction. Numerals indicate number of observations. Average weight of gelatin-treated animals autopsied on various days was: 2nd day, 46 gm., 3rd day, 48 gm., 7th and 8th days, 58 gm. Data from series 4.

possibly because the animals were autopsied at an earlier age than most, it was found that the gelatin solution had not been as completely absorbed as usual, and fluid was present in the peritoneal cavities of many of the rats for 2 or 3 days after the final injection. This continued absorption probably accounted for the further increase in relative kidney size after injections were discontinued. The chart demonstrates that a prompt increase in the relative size of the kidneys of the gelatin-treated animals occurred, followed by a prompt return toward normal as soon as the injections were discontinued and the gelatin was all absorbed from the peritoneal cavity. Evidence of some degree of renal damage was observed in the kidneys of several rats of this group, as will be discussed in more detail later.

Chart 3 shows the changes in the kidney weight-body weight ratios, during

the 10 day period following termination of a 9 day injection period, in animals receiving various protein solutions. The final injection is indicated by vertical arrow. Protein solutions had been almost completely absorbed when the first animals were autopsied on the 10th day.

The decrease in kidney weight-animal weight ratio after termination of protein injections, particularly when observations were made over a considerable period, was due in part to a dilution of the kidney weight increment which resulted from the injections, due to normal growth of the kidneys. In addition to this effect, however, there was a prompt decrease in the absolute difference

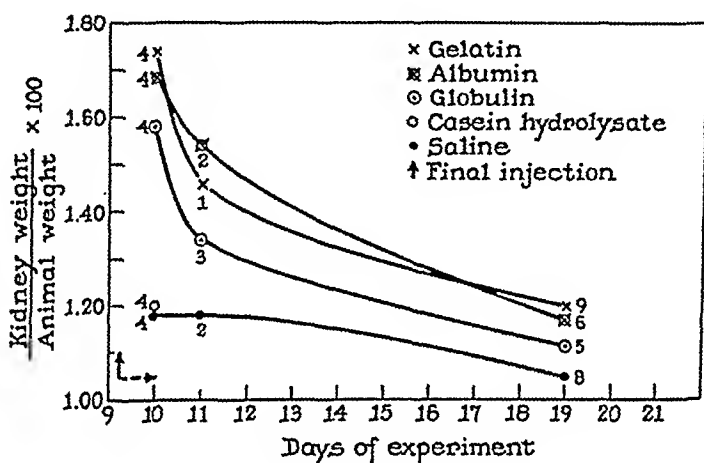


CHART 3. Showing return of kidneys of protein-treated animals toward normal size, following termination of a 9 day injection period. Data are from series 6. Numerals on chart indicate number of observations at each point. Horizontal arrow indicates duration of presence of protein solutions in peritoneal cavities of animals. Average weights of various groups of animals autopsied at different points on chart were: 10th day, 51 to 52 gm., 11th day, 52 to 56 gm., 19th day, 75 to 86 gm.

in size between the kidneys of protein-treated and control animals. Charts 2 and 3 indicate that some residual enlargements of the kidneys of the protein-treated groups probably remained at the end of the periods of observation. In series 1, there appeared to be slight enlargement of the kidneys of the gelatin-treated animals 2 months after the injections were discontinued. However, since any residual enlargement becomes a progressively smaller portion of the total renal tissue as the animals grow older, and greater variation in animal size occurs, the long term observations on small groups of animals were of doubtful significance. It can only be concluded that there was a prompt return of the kidneys of the protein-injected animals in the direction of normal size, after protein injections were discontinued.

Composition of Kidneys of Animals Receiving Various Solutions.—After it was apparent that injections of protein solutions resulted in renal enlargement,

it seemed of importance to determine whether the enlargement was due simply to an increase in the water content of the renal tissue, or whether it was due to an increase in material of approximately the same solid content as normal renal tissue. Water determinations were used to answer this question. Since the enlargement appeared to involve chiefly the cortical portion of the kidney, cortical tissue was used for the determinations. It is evident from data in Table V, representing water determinations on kidney tissue from a total of 70 animals, that only a slight part, if any, of the enlargement was due to an increase in the proportion of water to solids in the renal tissue of the protein-treated animals. The increase in weight was due to addition of water and solids in almost the same proportion present in normal kidney tissue. There was a slight increase in average relative water content of the tissue from animals

TABLE V
Moisture Content of Renal Cortical Tissue of Rats from Series 3, 6, and 7

Injected solutions*	Moisture	
	18 hrs. after final injection	10 days after final injection
	<i>per cent</i>	<i>per cent</i>
Saline	77.2	77.3
Urea	76.8	—
Casein hydrolysate	76.8	—
Gelatin	78.0	77.2
Albumin	78.8	78.3
Globulin	76.9	77.5
Serum	77.5	—

Injections were continued for 9 days to 2 weeks.

* Solutions previously described in text.

receiving albumin, and possibly in that of those receiving gelatin, 18 hours after termination of the injections. The 10 day values are less reliable because of the smaller number of determinations and greater spread of individual values.

Total nitrogen determinations on a few kidneys of various groups gave approximately similar results in most of the control and protein-treated animals and indicated that the protein concentration was probably normal in the protein-treated animals. The increase in kidney weight was apparently due in most instances to addition of water and protein in nearly the same ratio existing in normal kidneys.

Injected Protein in Renal Tissue.—Homogenates of renal cortical tissue from gelatin-injected animals were usually found to contain definitely more material resembling gelatin, in that it was precipitated by tungstate but not by a final concentration of 5 per cent trichloroacetic acid, than was found in the renal tissue from other groups. This was true even when the determinations were made several days after gelatin injections were discontinued. However, efforts

were not made to insure quantitative extraction of gelatin or other specific proteins from the renal tissue and quantitative determinations of gelatin were not made in most cases. The desirability of further studies was evident when, during the course of the experiments, it was found that kidney tissue from *control* animals sometimes contained more than the minimal amounts of "gelatin" observed in the early experiments.

In extracts of kidneys from rats that received human albumin and bovine globulin, serological precipitin tests showed the presence of these injected proteins. The amounts of extracted human albumin were much smaller than necessary to account for the total enlargement of the kidneys associated with injections of that protein, and it was not established beyond question that the results could not be accounted for on the basis of human albumin in the blood and urine retained by the renal tissue. Quantitative determinations of the globulin were not attempted.

These results were regarded as suggestive but not conclusive evidence that the renal parenchymal cells contained quantities of the injected proteins.

Microscopic Observations.—No abnormalities or differences were noted on microscopic examination in the kidneys of animals receiving saline, (Fig. 4), urea, or protein hydrolysate.

Kidneys from animals receiving gelatin showed, in addition to the enlargement and paleness apparent on gross examination, rather marked alterations in the cells of the convoluted tubules, particularly in the outer two-thirds of the cortex (Figs. 5 and 6). The proximal tubules were probably more markedly involved than the distal segments. The cells of the convoluted tubules were enlarged and contained what appeared to be clear spaces in the cytoplasm. These clear spaces were observed in sections of tissue that had not been exposed to aqueous solutions, as well as in those subjected to the routine staining procedures. There was no increase in fat content of the cells as shown by fat stains. The lumina of the convoluted tubules appeared to be decreased in width and in some cases they were almost closed, due to encroachment by enlarged cells.

Although the kidneys of the animals receiving solutions of albumin and globulin were definitely enlarged in the gross, on microscopic examination no definite and consistent changes were noted which might have been responsible for the enlargement. There appeared to be slight cytoplasmic alterations in the cells of the convoluted tubules in some cases. Increases in size of the cells of the convoluted or other tubules, or changes in caliber of the tubular lumina, if present, were not of sufficient magnitude to be recognized, and there was no other apparent explanation for the increase in bulk of the cortical tissue. In most of the animals of the protein-treated groups, no changes suggestive of an increased production of new cells in the cortex, were observed; mitotic figures were noted in the tubules of some animals autopsied early in the course of gelatin injections.

In the kidneys of some animals receiving proteins, protein material was visible in occasional glomerular capsules and tubular lumina. Rare isolated tubules in the region of the straight segments appeared to be plugged with protein material but similar tubules also were seen from time to time, but perhaps less frequently, in control kidneys. No abnormalities were noted in glomeruli, blood vessels, or interstitial tissue, even in animals treated and observed for prolonged periods. No changes suggestive of sensitization of the animals to the injected proteins were observed.

Three or four gelatin-injected animals of series 4—the series in which absorption of the injected 3 per cent gelatin solution was unusually slow—differed further from most of the protein-treated animals in that definite evidence of renal damage was observed on microscopic examination of the kidneys. These animals in which definite damage was recognized were among those which were autopsied from 2 to 5 days after gelatin injections were discontinued. The morphological alterations were much more extensive in the kidneys of one of the animals (Figs. 7 and 8) than in those of the others. These alterations were localized principally in the inner cortical and outer medullary zones where glomerular capsules and tubules appeared dilated and contained some protein material, and the tubules were lined by flattened epithelium containing mitotic figures, large hyperchromatic nuclei, and basophilic cytoplasm. Similar dilation of glomerular capsules and tubules, not accompanied by evidences of tubular necrosis or regeneration, was present at the time of autopsy in the kidneys of some of the gelatin-treated animals of series 9, particularly those receiving the more concentrated solution. In the kidneys of the majority of animals receiving each of the 3 per cent protein solutions, no changes were observed which could be considered evidence of renal damage, unless the cytoplasmic alterations in the tubular cells induced by gelatin are interpreted as evidence of damage.

In the kidneys of animals receiving gelatin, the appearance of the cells of the convoluted tubules gradually returned toward normal after injections were discontinued, with the clear spaces decreasing in size and finally disappearing. While there was considerable individual variation, the morphological alterations largely disappeared in most cases within about a week, during which time the renal enlargement and the "gelatin" in the renal tissue also decreased.

During the periods following the injections no impressive renal cytologic changes were observed in the animals receiving the control solutions, or those receiving albumin or globulin.

No consistent microscopic changes were observed in the livers of any of the groups of protein-treated animals.

Observations on Animals Treated with Rat Serum.—Since renal enlargement and an increase in urinary protein excretion were induced by injections of heterologous proteins, it was desirable to determine whether similar effects would result from injections of homologous proteins. The experiments with

the proteins already discussed, particularly the rather preliminary studies on the specific proteins in the urine, suggested that the increased proteinuria was not dependent on the heterologous nature of the injected proteins but would be produced by homologous proteins as well. Because purified rat proteins were not available, it was decided to investigate the effects of injecting homologous serum. Some of the data from these studies have been included in the tables and charts already presented, in order to facilitate comparisons with data from other groups. However, for several reasons, separate discussion of the experiments with rat serum seemed desirable.

In the first experiment with rat serum (series 7 of Table I), the animals with the possible exception of one, appeared quite well throughout the experimental period. Some enlargement of the kidneys occurred (Chart 1), but this was of less magnitude than with the other protein solutions. At the time of autopsy, more than 12 hours after the final injection, large amounts of unabsorbed fluid were found in the peritoneal cavities of some of the animals. In experiments where solutions of gelatin, albumin, or globulin had been injected for similar periods of time, absorption had nearly always been more complete. In addition, the serum-injected rats showed evidence of peritoneal irritation with definitely thickened renal capsules, not observed in the animals receiving the 3 per cent solutions of single refined proteins. Furthermore, urine collections made on the last 2 days of the experiment, which were the only specimens collected, showed no definite increase in protein content as compared with control specimens.

It was thought that perhaps ether retained in the rat serum (the donor animals were deeply anesthetized with ether) caused progressive peritoneal irritation with a corresponding decrease in the rate of absorption of injected serum, and that this explained the fluid retention in the peritoneal cavities, the low levels of urinary protein excretion, and the absence of greater renal enlargement. A second experiment (series 8) was then performed, using rat serum which was collected from donor animals after they were rendered unconscious by anoxia in a nitrogen chamber. In this experiment, the serum-treated animals retained much of the injected serum solution in the peritoneal cavities from the beginning, excreted little urine, and became definitely ill with elevated blood urea levels. Two of the four animals died on the 4th day after becoming quite pale and cold. The other two animals were autopsied and found to have greatly enlarged kidneys. Urine collected on the 3rd and 4th days from animals of this group contained considerably increased protein levels.

Microscopic Observations on Serum-Treated Animals.—Sections from the kidneys of all but one of the animals of the first serum-treated group appeared entirely normal, with the exception perhaps that the tubular lumina contained more protein material than normal. Changes similar to those observed in the gelatin-treated animals of series 4 with the severer forms of injury, were seen in milder degree in sections of the kidneys of one rat of this first group.

When the greatly enlarged kidneys of the second serum-treated group were sectioned, a discrete band occupying the region about the corticomedullary junction, which obviously contained large amounts of calcium (Fig. 9), could be seen with the unaided eye. Microscopic examination of sections confirmed the presence of extensive tubular damage with necrosis and calcification in the zone including the deepest portion of the cortex and adjacent medullary tissue (Figs. 10 and 11). Many glomerular capsules contained protein precipitates, and the tubules in the area beneath the zone of calcification were dilated, lined by flattened epithelium, and contained large amounts of deeply staining protein material.

DISCUSSION

Only the inconclusive data of Bordley and Richards (15) and Walker *et al.* (16) are available concerning the protein content of the normal glomerular filtrate, though the constant occurrence under uniform conditions of fairly uniform levels of protein in the urine of the normal rat (17) appears to be presumptive evidence that the glomerular filtrate of that animal contains some protein. Accumulated evidence (18-21) indicates that the glomerulus is at least the chief source of the protein which appears in the urine under the various abnormal conditions which have been studied; it does not eliminate the possibility, however, that all or a part of the protein in the urine under certain circumstances may be present as a result of defective tubular reabsorption of protein normally or abnormally filtered from the glomeruli (22). It was evident from examination of the sections in the present study that the increased proteinuria which occurred with the protein injections was associated with an increased amount of protein in the glomerular filtrate. Furthermore, gelatin appeared in the urine prior to the development of the characteristic changes in the tubular cells which were presumably associated with the presence of gelatin in the cells.

Because of the chronic nature of the experiments there may have been a compensatory increase in hemoglobin levels of the protein-injected animals, and the hemoglobin levels at the end of the injection periods may not have adequately reflected the magnitude of the blood volume changes.

Proteinuria has been observed in dogs following injections of homologous plasma (23), and in human patients without renal disease following injections of homologous albumin (24). In the present experiments, an increase in urinary protein excretion was observed in some, but not in all the rats receiving homologous serum; interpretation of the results was complicated by the occurrence in some of the animals of incomplete absorption of fluid, oliguria, and renal damage.

The rather cursory study of the specific proteins in the urine of the rats seemed to indicate that increased amounts of homologous protein, as well as quantities of the heterologous protein, appeared in the urine following injec-

tions of human albumin and bovine globulin, which accumulated in the plasma, but not following gelatin which was passed into the tubular lumina to at least as great extent, but which caused less hemodilution than the other proteins. These observations might be interpreted as suggesting that the filtration of a protein present in the plasma may be increased, with no increase in the concentration of the protein, by changes in dynamics of the glomerular circulation associated with an increase in blood volume, though other explanations of the observations are conceivable.

Reversible renal enlargement, comparable in degree and rapidity of development to that observed in the present experiments following the injections of proteins, has been shown to occur in rats on diets containing high levels of various proteins, including casein (25-27). This renal hypertrophy under conditions of high protein diets has usually been considered a result of an increase in renal work associated with the metabolism and excretion of products of protein digestion. Some degree of renal enlargement due to feeding of urea itself has been observed by some investigators (28), but others have obtained essentially negative results (25). Renal injury also has occurred under certain circumstances with high protein diets (29, 30).

In the present study, renal enlargement such as occurred with the protein injections, was not observed following injections of urea or casein hydrolysate. It perhaps is possible, in spite of careful planning of the experiments, that differences in food intake, nutritional state, or hydration of tissues, might have been responsible for the difference in relative renal size which developed in the animals receiving protein hydrolysate, as contrasted with those receiving protein itself. However, there was no evidence from examination of the animals that such differences were present to an important degree.

Another explanation of the occurrence of renal enlargement with injections of proteins but not with injections of protein hydrolysate or urea, which must be considered, is that the renal enlargement might have been caused at least in part by effects on the kidney of protein molecules *per se*, perhaps more specifically by effects on the tubular cells of the increased amount of protein filtered through the glomeruli, rather than entirely by effects of products of protein digestion and metabolism reaching the kidney through the blood stream. It might be pointed out that an elevation in urinary protein excretion has been observed incidentally accompanying an increase in the dietary protein level in rats (30, 31); however, it is not intended to suggest that the increase in proteinuria was the cause of the renal hypertrophy which occurred with the high protein diets. It is difficult to understand why the protein hydrolysate injections in the present experiments did not induce some increase in proteinuria and renal enlargement through the same mechanisms as were involved with increasing the protein content of the diet.

Morphological observations by a number of European investigators, and

more recent studies by Oliver (21), Smetana and Johnson (32), Smetana (33), and Rather (34), have provided fairly conclusive evidence that at least certain proteins present in the fluid passing through the tubules may be reabsorbed by the tubular cells and accumulate to some extent within these cells. However, the results of Bott and Richards (20) indicated that not more than a small portion of the filtered protein was reabsorbed under the conditions of their experiments. The reabsorbed protein under normal conditions presumably is digested by the tubular cells (34). The observations of others on protein reabsorption, together with our own morphological observations, particularly with gelatin which was the only protein seeming to produce recognizable enlargement of the tubular cells, suggested that the renal enlargement might have been caused in some part by enlargement of individual tubular cells as a result of reabsorption and temporary accumulation within the cells of protein passing through the glomeruli, together with water associated with it. The cytoplasmic changes produced by gelatin were similar to those described by Popper (35) and Skinsnes (36) in human kidneys following gelatin administration. Furthermore, an increase in metabolic activity of the renal cells associated with an increased reabsorption and degradation of protein might have been responsible for some degree of enlargement of the kidneys.

An increase in volume of intraluminal material within the renal tubules very probably was present in a few animals in which severe renal injury occurred, and in some other gelatin-treated animals particularly during the early periods of the injections, but it appeared unlikely that changes of this nature were responsible for the renal enlargement which regularly followed the protein injections.

In the majority of animals receiving each of the 3 per cent protein solutions, there was no evidence of gross disturbances of renal function. Renal clearance studies have not been done, though such studies might yield interesting results. The changes in renal size and the alterations in the appearance of the convoluted tubules were regarded for the greatest part as reversible morphological expressions of exaggerated physiological processes, and no inflammatory, necrotic, or sclerotic changes were observed in the kidneys, either during or following the injections. Urinary protein excretion promptly returned to normal levels after injections were discontinued. It was concluded that under the conditions of the experiments, prolonged continuous proteinuria of the degree obtained with these injections in most cases did not lead to a persistent increase in glomerular permeability or to any other form of chronic or progressive renal injury.

Definite evidences of tubular injury of a severe degree were observed, however, in a few animals receiving 3 per cent gelatin, and dilatation of glomerular capsules and tubules was present in some of the animals of series 9 receiving gelatin. Whereas most of the gelatin deposition presumably occurred in the tubular cells in the outer two-thirds of the cortex, the zone of injury was about

the corticomedullary junction. Whether this injury was a result of plugging of the tubules, or was due to some other action of gelatin, or was unrelated to any effects of gelatin on the kidney, was not determined with certainty. While the destruction of cells and proliferative changes in the dilated tubules might be considered evidence against mechanical plugging alone as the cause of the tubular lesions, at least the damaged tubules in most cases contained more protein within the lumina than usually was observed in undamaged tubules. Urine from the animals in which severe injury occurred with gelatin, unfortunately was not collected or examined. The possibility that these animals became dehydrated, due to failure to absorb part of the injected fluid, or due to failure for some reason to drink water after the injections were discontinued, in the case of the animals of series 4 which were not autopsied for several days after injection, and that dehydration contributed to the production of the injury,—as has been observed with hemoglobin (37),—must be considered. It should be noted that the concentration of protein in the urine of most of the animals in the present experiments, particularly those receiving albumin and globulin, and those of the first group receiving serum, was not as great as is often observed clinically. Furthermore, in animals with already diseased kidneys, the effects of protein injections and increased proteinuria might have been quite different from the effects observed in the present experiments employing animals with normal kidneys.

Before examining the kidneys of the second series of rats receiving homologous serum, it was considered likely that the erratic absorption of serum, and the oliguria or anuria which occurred, and perhaps also the large amounts of protein in the urine, were due to shock rather than to primary renal damage. However, the remarkable lesions which were observed were not similar to any lesions which have been attributed to disturbances of the renal circulation accompanying shock. Tubular plugging may have played a part in the production of the lesions; both the tubules and the glomerular capsules often contained large amounts of protein. The lesions were similar in certain respects to those which occurred in some of the rats receiving gelatin. Because of the great difference in the results obtained in the first and second experiments with rat serum, the profound anoxia to which the donor animals in the second experiment were subjected warrants further investigation as the possible cause of the noxious effects of the serum. It may be that the injury was due to bacterial or chemical contamination of the serum or changes in the serum subsequent to collection, and, until an attempt has been made to repeat the results, no conclusions concerning the etiology of the lesions are justified.

Finally, it might be noted that the kidneys of the protein-treated animals of the present experiments, particularly those receiving gelatin, bore certain morphological resemblances to "nephrotic" kidneys. These similarities in appearance suggested the *possibility* that, as has been concluded by a number

of investigators, certain of the changes which are seen in kidneys in diseases characterized by high levels of protein in the urine may be secondary alterations due to excessive amounts of protein passed through the glomerular membranes. Obviously no conclusions regarding the nature or the site of the fundamental disturbances in nephrosis can be drawn from these observations.

SUMMARY

Repeated intraperitoneal injections twice daily of various proteins into young rats were regularly accompanied by an increase in the protein content of the urine, significant renal enlargement, and often some degree of renal pallor. The most marked changes were induced by gelatin, followed in order by human albumin and bovine globulin. Rat serum produced similar but less conclusive changes. Similar changes were not produced by equivalent amounts of urea or casein hydrolysate.

In sections from the kidneys of animals receiving gelatin, the cells of the convoluted tubules appeared enlarged, and they contained clear "spaces" throughout the cytoplasm. The tubular cells of the animals receiving the other solutions were not obviously altered in size or shape, and the cytoplasmic changes were slight or absent. There was little evidence of increased multiplication of cells or of tubular dilatation in the kidneys of any of the groups.

Changes in concentrations of plasma proteins and hemoglobin, and the results of preliminary studies of the injected proteins in urine and renal tissue following the injections, are described and their possible significance discussed.

It appears that the renal enlargement, as well as the increase in proteinuria and the tubular alterations which followed the protein injections, might have been caused in part by effects on the kidney of protein molecules *per se*, perhaps most likely by the effects on the tubular cells of an increased amount of protein filtered through the glomerular membranes, rather than entirely by effects of products of protein digestion and metabolism reaching the kidney through the blood stream.

In the majority of animals there was no evidence from the morphological or functional studies, that the prolonged and continuous proteinuria induced by the protein injections resulted in renal damage, unless the renal enlargement, and the cytoplasmic changes which occurred regularly with gelatin, are considered evidence of damage. Renal enlargement and proteinuria promptly regressed after injections were discontinued.

Lesions characterized by severe degrees of tubular damage, possibly as a result of tubular plugging, were observed in some of the animals of one group receiving gelatin solution of the usual concentration, and dilatation of renal tubules and glomerular capsules was present in some other gelatin-treated animals autopsied after relatively brief injection periods. A description is also presented of lesions

of remarkable character which developed in the kidneys of all the animals of one small group receiving homologous serum obtained from severely anoxic donors.

The possible relationship between the renal changes in the protein-injected animals and certain alterations of the kidneys observed in diseases characterized by large amounts of protein in the urine, is considered.

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EXPLANATION OF PLATES

PLATE 32

The photographs were made by Mr. R. F. Carter.

FIG. 1. Comparing left kidney from gelatin-treated rat (above) with homolateral kidney from comparable saline-injected control animal (below). The organs had not been fixed. The animals were from series 1 and received injections for 6 weeks. Note enlargement, particularly in the cortical portion, and paleness of the kidney from the gelatin-injected animal. Weights are as follows:—

$$\text{Control rat: weight of kidneys, 1.85 gm.; weight of rat, 146 gm.; } \frac{\text{Weight of kidneys}}{\text{Weight of rat}} \times 100 = 1.27$$

$$\text{Gelatin rat: weight of kidneys, 2.50 gm.; weight of rat, 140 gm.; } \frac{\text{Weight of kidneys}}{\text{Weight of rat}} \times 100 = 1.79$$

Magnification $\times 2$.

FIG. 2. Comparing left kidney from albumin-treated rat (above) with control kidney (below). The animals were from series 2, and received injections for 5 weeks. Note enlargement and cortical thickening in kidney of albumin-treated animal. Weights are as follows:—

$$\text{Control rat: weight of kidneys, 1.54 gm.; weight of rat, 126 gm.; } \frac{\text{Weight of kidneys}}{\text{Weight of rat}} \times 100 = 1.22$$

$$\text{Albumin rat: weight of kidneys, 2.10 gm.; weight of rat, 126 gm.; } \frac{\text{Weight of kidneys}}{\text{Weight of rat}} \times 100 = 1.67$$

Magnification $\times 1.5$.

FIG. 3. Comparing right kidneys from comparable rats of series 6 receiving various solutions. Animals received injections for 9 days. Top row, left to right:

$$\text{Gelatin: weight of kidneys, 1.10 gm.; weight of rat, 64 gm.; } \frac{\text{Weight of kidneys}}{\text{Weight of rat}} \times 100 = 1.72$$

$$\text{Albumin: weight of kidneys, 1.03 gm.; weight of rat, 66 gm.; } \frac{\text{Weight of kidneys}}{\text{Weight of rat}} \times 100 = 1.56$$

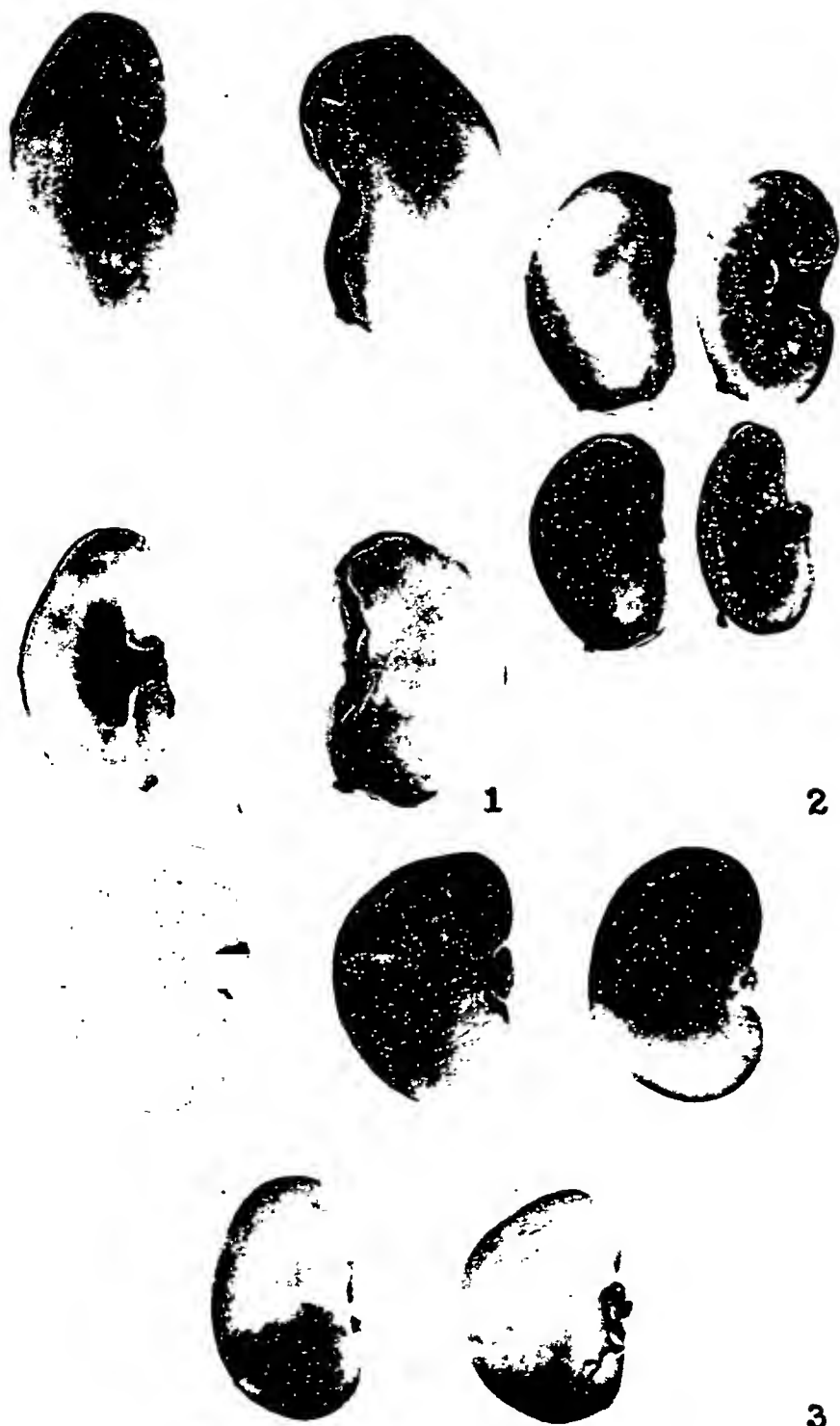
$$\text{Globulin: weight of kidneys, 1.03 gm.; weight of rat, 66 gm.; } \frac{\text{Weight of kidneys}}{\text{Weight of rat}} \times 100 = 1.56$$

Bottom row, left to right:

$$\text{Casein hydrolysate: weight of kidneys, 0.78 gm.; weight of rat, 64 gm.; } \frac{\text{Weight of kidneys}}{\text{Weight of rat}} \times 100 = 1.22$$

$$\text{Saline: weight of kidneys, 0.78 gm.; weight of rat, 64 gm.; } \frac{\text{Weight of kidneys}}{\text{Weight of rat}} \times 100 = 1.22$$

Magnification $\times 4$.



(Baxter and Cotzias: Parenteral protein and the kidney)

PLATE 33

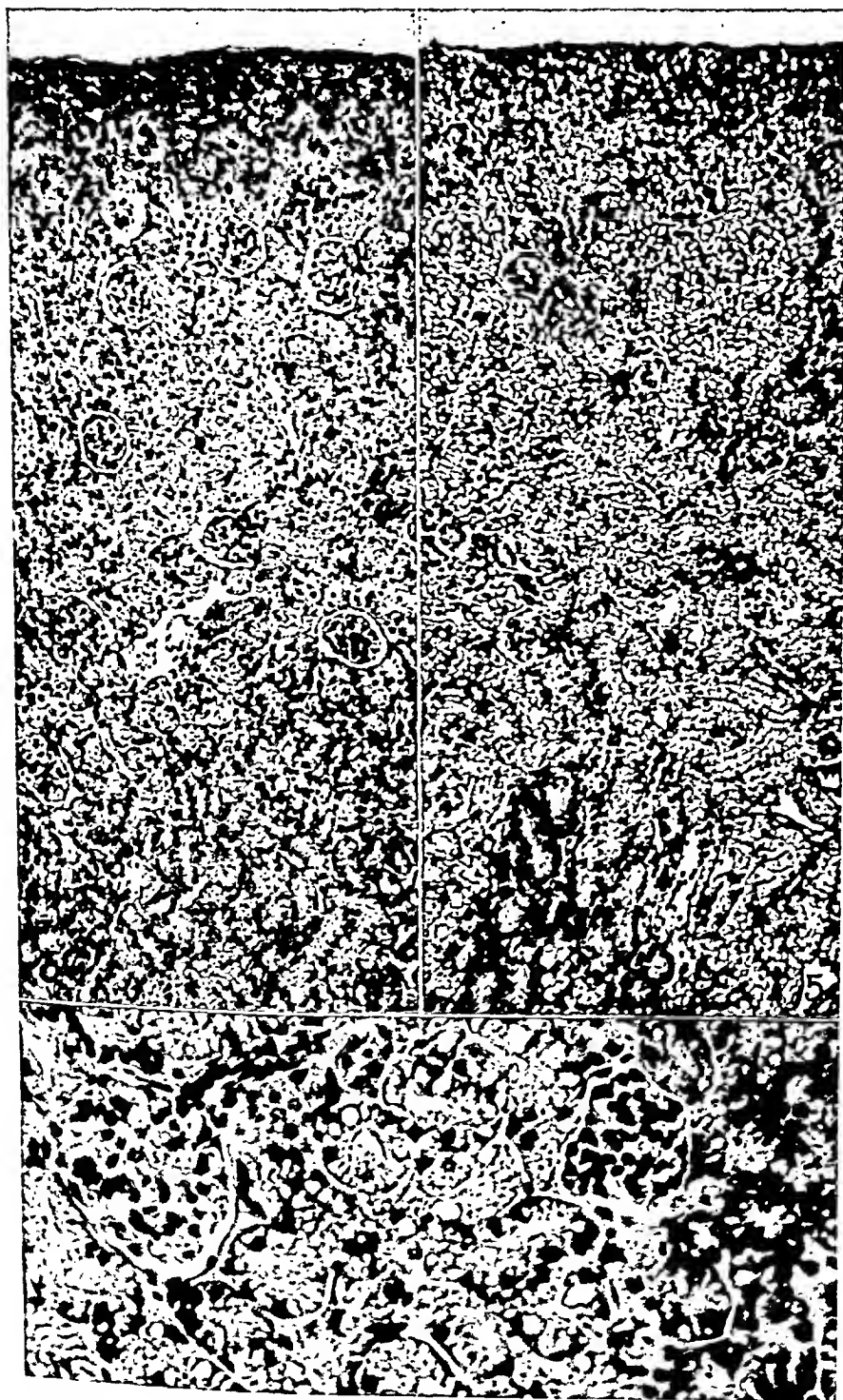
All the sections were stained with hematoxylin and eosin.

The photographs were made by Mr. J. A. Carlile.

FIG. 4. Sagittal section through a control kidney of same group as one shown in Fig. 1. $\times 120$.

FIG. 5. Similar section through kidney of comparable gelatin-treated animal of series 1. Note enlargement of cells of convoluted tubules, and what appear to be clear spaces within the cytoplasm of the cells. $\times 120$.

FIG. 6. Higher magnification of section shown in Fig. 5, showing cytoplasmic alterations in greater detail. $\times 350$.



(Baxter and Cotzias: Parenteral protein and the kidney)

PLATE 34

All the sections were stained with hematoxylin and eosin.

The photographs were made by Mr. C. F. Reather, Johns Hopkins School of Medicine.

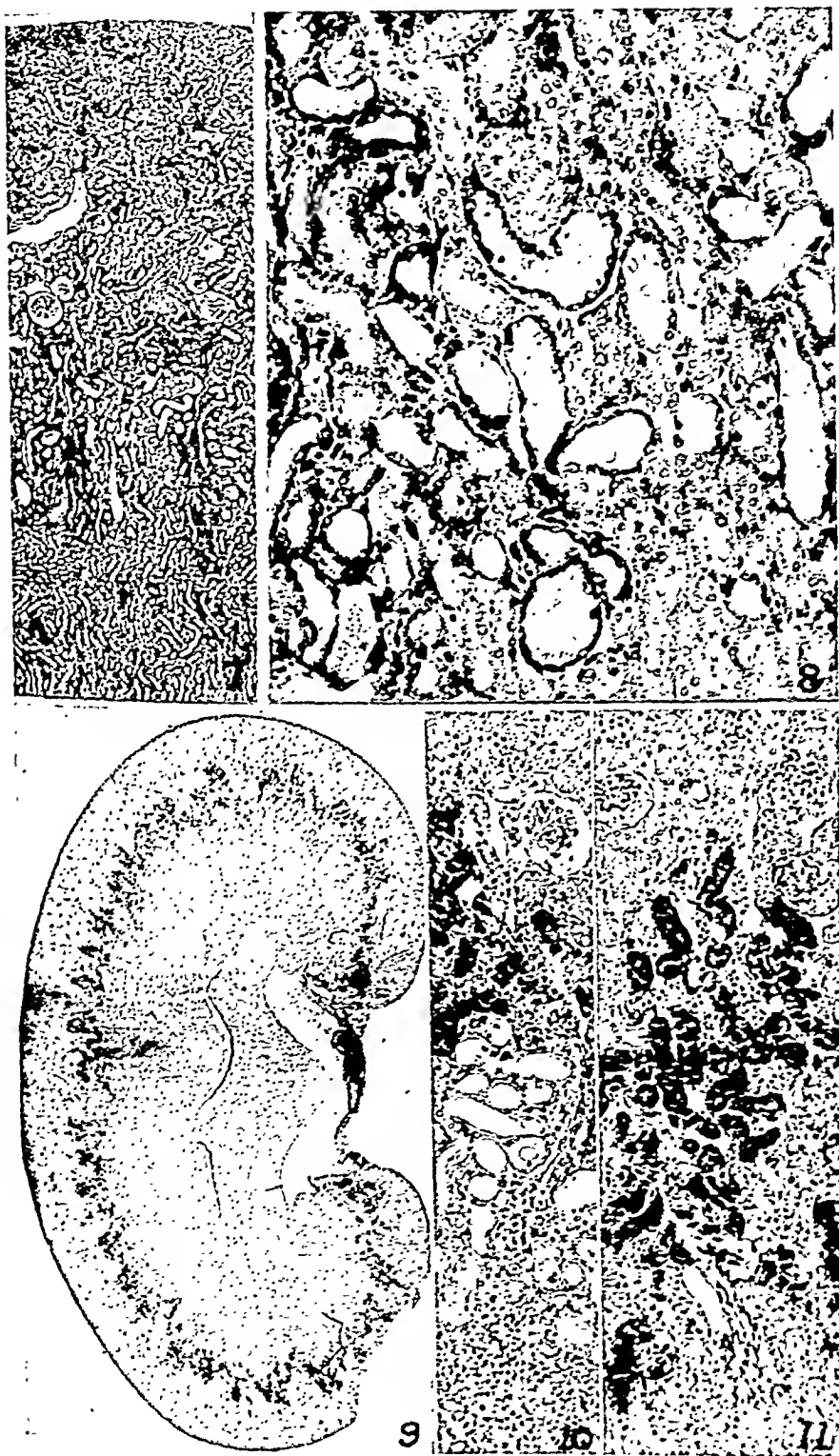
FIG. 7. Sagittal section through kidney of gelatin-treated animal of series 4. This animal received only three injections of gelatin at 12 hour intervals, and was autopsied 54 hours after the last injection. At a higher magnification clear spaces could still be seen in the cells of the convoluted tubules in the outer two-thirds of the cortex. Deeper in the cortex and in the outer portion of the medulla, many glomerular capsules and tubules are dilated. This section shows the most extensive damage observed in any of the kidneys of the animals receiving the 3 per cent solution of gelatin. $\times 40$.

FIG. 8. Higher magnification of the section shown in Fig. 7, showing details of the tubular alterations in the zone of the most extensive injury. Many of the tubules are dilated, lined by flattened and markedly basophilic epithelium, and contain pink-staining protein material. Mitotic figures and other nuclear changes indicative of active cellular proliferation were also present. $\times 200$.

FIG. 9. Sagittal section from kidney of a serum-treated animal of series 8, which became obviously ill and was autopsied on the 5th day. This animal was anuric through a considerable part of the injection period but excreted a moderate amount of urine containing large amounts of protein during the 24 hours before autopsy. The blood urea level was approximately 100 mg. per cent at the time of autopsy. The zone of tubular necrosis and calcification, and the zone of tubular dilatation, are shown well. $\times 8$.

FIG. 10. Higher magnification of section shown in Fig. 9. The zone of calcification can be seen about the region of the corticomedullary junction. Adjacent to and toward the pelvis from this calcified zone, there is the zone of dilated tubules, many of which contain protein. Many tubules far down toward pelvis, which were not dilated, also were full of protein material. $\times 100$.

FIG. 11. A similar section from kidney of the other serum-treated animal of series 8 which was autopsied on the 5th day. In this kidney most of the glomerular capsules contained protein precipitates. $\times 100$.



(Baxter and Cotzias: Parenteral protein and the kidney)

SEROLOGICAL STUDIES IN RHEUMATIC FEVER

I. THE "PHASE" REACTION AND THE DETECTION OF AUTOANTIBODIES IN THE RHEUMATIC STATE

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It has been suggested that an allergic type of mechanism may be responsible for the rheumatic process. However, it must be recognized that the evidence for this is far from conclusive. The allergic hypothesis of the disease is based chiefly upon certain characteristics of the disease which suggest allergy (5) such as the latent period after infection with *S. hemolyticus* (1-5), the clinical similarity to serum sickness (6), and the morphological analogy between rheumatic lesions and those produced by necrotizing allergic reactions in experimental animals (7-11). It is generally recognized that infection with the hemolytic streptococcus is frequently followed by the appearance of circulating antibodies (12, 13) or skin sensitivity (14-16) to streptococcus products. However, these responses do not appear to differ qualitatively or quantitatively in rheumatic subjects from those of non-rheumatic individuals with streptococcus infections, particularly if the infection is persistent (17-19). Identification of the antigens or antibodies involved in the presumed allergic reaction in rheumatic fever would give experimental support to the allergic hypothesis. There are relatively few reported studies concerned with the demonstration of antibodies in rheumatic patients which are not found in other individuals recovering from streptococcus infections. Thus, the phase reaction reported by Coburn and Pauli (20), and the development of autoantibodies to liver tissue (Brokman, Brill, and Freundel (28)) and to heart tissue (Cavelti (30)) have been advanced as serological phenomena peculiar to the rheumatic state, and possibly related to the necrotizing allergic reaction presumed to occur. The present study is concerned with an attempt to repeat and, if possible, to extend previous investigations indicating the existence of allergic mechanisms in rheumatic fever.

A. The Phase Reaction

In 1939 Coburn and Pauli (20) reported that a substance called a "precipitinogen" appeared in the serum of a rheumatic subject following a sore throat which precipi-

* This work was done during the tenure of a Life Insurance Medical Research Fellowship. Aided in part by the Masonic Foundation for Medical Research and Human Welfare.

tated when mixed with the serum taken during the subsequent rheumatic attack. Because the reaction occurred with serum obtained during phase I or II (the sore throat and the latent periods), when mixed with serum from phase III (the period of rheumatic activity), it has been called the "phase reaction." If a rheumatic attack did not develop following the sore throat, it was reported that the precipitinogen was not present, and no precipitin developed. When the rheumatic attack ran a polycyclic course, the precipitinogen and the precipitin alternately appeared in the serum. It was suggested that this precipitinogen might represent a "secondary antigen" derived from a combination of streptococcal products and human tissue constituents. Although the phenomenon did not necessarily represent an antigen-antibody reaction, it appeared to be one, and one intimately associated with the occurrence of rheumatic fever.

Despite the apparently fundamental significance of this reaction, there were no further reports concerning it until 1946 when Wedum and Wedum (21) seemed to have confirmed some of the observations of Coburn and Pauli. However, there are certain differences between the two reports which would appear to be incompatible with the hypothesis suggested by both groups of investigators. Wedum and Wedum found that their presumed antigen occurred during the first few days *after* the onset of a rheumatic attack, not preceding it, and again appeared at the end of the attack in contrast to Coburn's observations. Occasionally both substances, *i.e.* precipitinogen and precipitin, were said to coexist in the same serum sample, because a fine precipitate occurred when the unmixed sample of serum was incubated (21). Wedum and Wedum also found that the phenomenon was not peculiar to rheumatic fever. It occurred in uncomplicated nasopharyngitis, in primary atypical pneumonia, and in a few miscellaneous conditions. Unfortunately, much of this work was done with the sera of different individuals; that is, serum from one subject with a sore throat was mixed with that of another subject who had rheumatic fever.

Experimental.—Sera were obtained from patients on the wards and in the Rheumatic Fever Follow-up Clinic of the Presbyterian Hospital, the Babies Hospital, and The Pelham Home for Children. The latter institution, a convalescent home, offered an opportunity to obtain sera from known rheumatic subjects before the onset of a sore throat or of a rheumatic recrudescence. Sera were obtained at weekly intervals, generally more frequently during active illness, and less frequently during late convalescence and quiescent periods. Usually sera taken at different times from the same individual were used for one series of tests. In a number of instances sera from these patients were intermixed with no alteration in results.

The tests were performed using the technique previously described (20). 0.1 ml. of each serum to be tested was mixed with an equal quantity of another sample from the same patient. The mixture was incubated at 37°C. for 2 hours, and put in the ice box overnight. After centrifugation the next morning, tubes were agitated and examined with a lens. Particulate matter, scarcely visible to the unaided eye, was the strongest positive reaction obtained, as in the original work. Readings were graded as \pm , +, and ++, and independent readings by each author gave little significant variation. Unlike the previous work, controls of test serum alone, and test serum plus normal serum, were used in addition to the serum plus saline controls. Every serum was cross-tested with every other serum from the same patient.

The sera tested included 221 samples from 38 cases of rheumatic fever (20 cases with only a single serum sample); 16 samples from 7 cases of streptococcus pharyngitis in non-rheumatic individuals; 85 sera from 11 cases of streptococcus pharyngitis in rheumatic subjects who did

not develop demonstrable rheumatic activity; and 21 sera from 4 miscellaneous cases—2 of "idiopathic" myocarditis, 1 of acute gonorrheal arthritis, and 1 of an asymptomatic inactive rheumatic subject.

The results of the tests are shown in Table I. From 18 active rheumatic patients followed for long periods of time (Table I, A), negative, irregularly positive, and uniformly positive precipitin reactions were obtained. When positive precipitin tests were obtained they were obtained with any combination of serum samples irrespective of their relationship to the phase of the disease. Indeed, many of these positively precipitating sera also showed positive reactions when mixed with control non-rheumatic sera. Table I, A illustrates the haphazard relationship of a negative or a positive precipitation test to the periods of sore throat, and to the course of active rheumatic fever. Table I, B and I, C presents the results of the "phase reaction" test in inactive rheumatic subjects and in normal (non-rheumatic) patients with sore throats not followed by attacks of rheumatic fever. Of the inactive rheumatic group (Table I, B) positive precipitation was noted with the sera from four patients, no precipitation occurred with the sera from four other patients; and doubtfully positive precipitation was noted with the sera from the remaining three individuals. There is a preponderance of negative precipitation tests in the non-rheumatic group with sore throats (Table I, C). It should be noted that fewer sera were obtained in these instances. Samples of serum from a case of gonococcal arthritis (Table I, D) were positive throughout. A completely inactive and asymptomatic subject with a history of rheumatic fever 3 years previously gave scattered weakly positive and doubtfully positive precipitation reactions. Therefore, no correlation was found between the occurrence of precipitates and the occurrence of any clinical episode, either sore throat or rheumatic fever. Furthermore, repeated tests failed to give reproducible results.

Further evidence against a possible antigen-antibody reaction occurring in these sera appeared from the following observations:

When one or both sera usually showing precipitates were diluted with an equal amount of saline, and mixed, no precipitation occurred. If either serum contained a true precipitin, and the other an antigen, slight dilution of one or the other of the reagents would not be expected to affect the formation of the precipitate.

Sera, showing precipitate, were mixed in various dilutions in the presence of complement. No fixation of complement occurred if amounts of serum below the anticomplementary concentrations were used. Certain known antigen-antibody systems do not fix complement. Therefore, this observation is only suggestive of the absence of an immune system.

Carminic acid particles (22) and collodion particles (32) were used in an attempt to make a microscopic reaction more visible. No agglutination of the mixtures occurred.

Serum obtained from one patient after a sore throat was injected intracutaneously during his rheumatic attack. No skin reaction occurred, either of the immediate or delayed type. Similarly an attempted Prausnitz-Küstner reaction in a normal individual with the sera containing the "precipitinogen" and "precipitin" gave negative results.

TABLE I
The Results of Precipitin Tests for the "Phase Reaction"

Patient	No. of sera	Periods during which sera were obtained	Results and comments on cross-precipitin tests in relation to course	Precipitin reaction with normal sera of same blood group
<i>A. Acute Rheumatic Fever</i>				
J. M.	18	(a) Initial sore throat. (b) Latent period, 1 mo. (c) Polycyclic rheumatic fever for 4 mos.	Slight precipitation scattered throughout	+
J. B.	11	(a) Early and polycyclic acute rheumatic fever (b) Quiescent convalescence over 4 mos.	Precipitation present in all tests	+
F. M.	6	(a) Very early acute rheumatic fever for 2 wks. (b) Quiescent convalescence for 3 wks.	Two sera at end of period of activity showed weak precipitation with each other and when alone. All others negative	-
A. T.	16	(a) Latent period 3 wks. (b) Early rheumatic fever. (c) Polycyclic course for 4 mos.	Scattered precipitation, particularly with one of latent period sera against all later sera and against controls	+
R. C.	3	(a) Acute rheumatic fever becoming normal by all usual criteria in 10 days	Weak precipitation between 1st and 3rd sera	-
B. V.	15	Acute rheumatic fever polycyclic for 3 mos.	All negative	-
A. G.	4	(a) Acute rheumatic fever for 1 mo. (b) Convalescent 1 mo.	First serum precipitates with remaining three but all four precipitate with control	+
C. D.	3	Acute rheumatic fever, 1 mo. duration	All sera form slight precipitates with each other, by themselves, and with controls	+
G. W.	4	(a) Acute rheumatic fever for 1 mo. (b) Quiescent for 1 mo.	Scattered precipitates in six of the tests but three are sera alone	+

TABLE I—Continued

Patient	No. of sera	Periods during which sera were obtained	Results and comments on cross-precipitin tests in relation to course	Precipitin reaction with normal sera of same blood group
<i>A. Acute Rheumatic Fever—Continued</i>				
W. H.	3	(a) Acute rheumatic fever for 2 wks. (b) Quiescent in 3rd wk.	All negative	—
R. A.	6	(a) Sore throat (b) Latent period for 2 wks. (c) Asymptomatic exacerbation for 4 wks. (ESR rising to 80)	All sera precipitate against each other	—
B. D.	19	(a) Asymptomatic 2 wks. (b) Sore throat 1 wk. (c) Latent period 3 wks. (d) Exacerbation 5 wks. (e) Quiescent 9 wks.	Scattered precipitations unrelated to course	+
H. H.	17	(a) Sore throat 1 wk. (b) Latent period 2 wks. (c) Active rheumatic fever 13 wks. polycyclic (d) Quiescent 5 wks.	Scattered weak precipitations—no relation to course	+
M. P.	18	(a) Sore throat 1 wk. (b) Latent period for 5 wks. (c) Acute rheumatic fever for 12 wks. (d) Quiescent for 6 wks.	As above	+
A. S.	24	(a) Asymptomatic 3 wks. (b) Tonsillitis 1 wk. (c) Immediate onset acute rheumatic fever which continued polycyclic for 15 wks. (d) Quiescent 4 wks. (e) ESR rising to 43 for 3 wks., then normal (f) 4 samples in next 4 mos.	As above	—
G. K.	13	(a) Subsiding acute rheumatic fever (b) Quiescent 2 wks. (c) Sore throat 1 wk. (d) Asymptomatic 3 wks. (e) Recurrent activity 3 wks. (f) Quiescent for 2 mos.	All tests showed weak precipitates	+

TABLE I—Continued

Patient	No. of sera	Periods during which sera were obtained	Results and comments on cross-precipitin tests in relation to course	Precipitin reaction with normal sera of same blood group
<i>A. Acute Rheumatic Fever—Continued</i>				
E. C.	11	(a) Subsiding acute rheumatic fever 6 wks. (b) Quiescent 14 wks.	As above	+
B. F.	17	(a) Rheumatic fever for 3 mos. (b) Quiescent for 1 mo. (c) Chorea for 1 mo.	Scattered positives with no relation to course	+
6 other cases of acute rheumatic fever—sera obtained at various times during the course—all gave a weak precipitate alone, with one another and with controls				+
<i>B. Inactive Rheumatic Subjects with Sore Throats Not Followed by Recrudescences</i>				
R. B.	7	Sore throat for 4 days. Elevated ESR for 2 wks. Quiescent for 6 wks.	All sera precipitate weakly with each other and with controls	+
I. R.	4	Sore throat	All negative	—
C. V.	3	Sore throat—followed for 2 wks. after	All negative	—
A. I.	4	Sore throat 1 mo. thereafter	All negative	±
C. S.	14	Followed 4 mos. at convalescent home, before, during, and after sore throat without rheumatic activity	Scattered precipitates unrelated to any period of observation	Occasionally positive
R. H.	10	5 mos. at convalescent home. One sore throat. No active rheumatic fever	All tests show precipitate formation	+
A. H.	9	As above	Scattered precipitates	+
L. L.	11	As above	All doubtfully positive	±
B. C.	6	As above	All doubtfully positive	+
P. C.	8	As above	All doubtfully positive	+
S. C.	9	As above but with two sore throats	All tests negative	—

TABLE I—*Concluded*

Patient	No. of sera	Periods during which sera were obtained	Results and comments on cross-precipitin tests in relation to course	Precipitin reaction with normal sera of same blood group
<i>C. Non-Rheumatic Subjects with Streptococcus Pharyngitis</i>				
G. D.	2	(a) Sore throat (b) Quiescent 2 wks. later	Doubtful precipitation	—
G. G.	2	Same but 18 days apart	Negative	—
H. H.	2	Same 12 days apart	Negative	—
B. G.	3	Sore throat, 5 days and 1 mo. later	Negative	—
T. M.	2	2 days apart	Negative	—
M. O'C.	2	13 days apart	Negative	—
R. S.	3	Sore throat, 3 days and 13 days later	Negative	—
<i>D. Miscellaneous Cases</i>				
J. R.	8	Old rheumatic subject. Asymptomatic and quiescent for 3 mos.	Scattered weak and doubtful precipitates	±
R. T.	3	3 wks. of gonorrheal arthritis	All tests precipitate including controls	+
W. M.	5	"Idiopathic" myocarditis 5 wks.	All tests negative	—
B. S.	5	Probable "idiopathic" myocarditis and pulmonary embolism 6 wks.	All tests negative	—

Serum of a patient which did show a tendency to form particulate matter with other sera, including control sera, showed the same tendency, if after incubation by itself, and centrifugation, the supernatant was again incubated and centrifuged. Therefore, the "reagents" could not be absorbed from the sera.

Discussion.—The "phase reaction," a serological phenomenon thought to be specific in rheumatic individuals has been cited as laboratory evidence for the hypothesis that an allergic mechanism is concerned with the development of rheumatic fever. This study has not corroborated previous reports. The inconstant nature of the formation of precipitates on repeating identical tests,

and the lack of visible formation of precipitates on dilution with saline, suggest that the particulate matter formed in these tests is not due to a specific immune reaction. It is perhaps not unlike the non-specific precipitation that occurs in uncontaminated sera which are allowed to stand for a period of time, even in the ice box. Sera cleared of particulate matter after prolonged centrifugation in the cold before the performance of a quantitative precipitin test (23) occasionally develop particulate matter after incubation at 37°C. This may result in a precipitate giving an appreciable amount of nitrogen in the serum control or "blank" tubes. There are a number of poorly understood changes in the sera of patients with rheumatic fever and certain other illnesses which are reflected in phenomena such as the erythrocyte sedimentation rate (25) and the "colloidal" tests (26). In addition, chemical changes are known to occur in active rheumatic sera (24, 27). These abnormalities may or may not be related to the non-specific precipitation that occurs in serum when it stands alone in the ice box or when it is mixed with another serum and incubated.

B. Autoantibodies in Rheumatic Fever

Autoantibodies and isoantibodies occasionally cause severe necrotizing allergic reactions, as in the Donath-Landsteiner reaction, erythroblastosis foetalis and, occasionally, cold hemagglutination. However, such antibodies may exist without obvious widespread tissue damage, as in the case of the Wassermann antibody. Therefore, the demonstration of an autoantibody in rheumatic fever may be of interest but does not *per se* establish the rôle of the tissue reaction in response to the autoantibody in rheumatic fever. The concept of an autoantibody in rheumatic fever was suggested by Brokman, Brill, and Freundzel (28). They found that sera from rheumatic patients fixed complement when mixed with an extract of liver obtained at the autopsy of a rheumatic child. Sera from other diseases did not fix complement with this "antigen." Unfortunately, anticomplementary controls were not reported. It is well known that tissue extracts are frequently anticomplementary, as are, occasionally, rheumatic or other sera in certain concentrations. Furthermore, Eaton and his associates reported that complement fixation occurs when liver tissue is mixed with sera from a variety of illnesses as well as with some normal sera (29).

Experimental.—Tests were made for a reaction similar to that of Brokman, Brill, and Freundzel. In the absence of fresh liver tissue, heart and lung tissue from a rheumatic individual, and placental tissue from a living rheumatic subject were used as "antigens." Eight sera, from active rheumatic subjects of the same blood groups as the individuals from whom the tissues had been obtained were mixed in various dilutions in the presence of 4 hemolytic units of complement. The traditional technique was employed, with 0.2 ml. of diluted "antigen," 0.2 ml. of diluted serum, and 0.2 ml. of complement. After incubation for a half hour, 0.2 ml. of a standardized suspension of sensitized sheep cells was added. No evidence of complement fixation was found in dilutions of serum and of tissue extract which were not anticomplementary alone.

Another autoantibody system was suggested by Cavelti (30). He found that one of four normal (*i.e.*, non-rheumatic) hearts used as antigen reacted strongly with sera from 27 of 36 rheumatics studied. By mixing the antigen with collodion particles, the presumed immune aggregation was made macro-

TABLE II

Results of the Collodion Particle Method for the Detection of "Autoantibodies" in the Sera of Rheumatic and Syphilitic Patients

Patient and serum No.	Antigens														
	M. (carcinoma)					W. (chronic nephritis)					H. (rheumatic)				
	Heart	Lung	Liver	Spleen	Kidney	Heart	Lung	Liver	Spleen	Kidney	Heart	Lung	Muscle	Spleen	Kidney
Rheumatic fever															
Zi. 1	0	+	+	0	0	0	0	0	0	0	0	+	0	0	0
" 2*	0	0	++	++	+	0	0	0	0	0	0	0	0	0	0
Pe. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	++	0
" 2	0	0	0	0	0	0	0	0	0	0	0	0	0	++	0
Ki. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	++	0
" 2	0	0	0	0	0	0	0	0	0	0	0	0	0	++	0
Va. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
" 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dr. 1	++	0	++	0	0	0	0	0	0	0	0	0	0	0	0
" 2	0	0	0	++	0	0	0	0	0	0	0	0	0	0	0
Du. 1	0	0	0	0	0	0	0	0	0	0	0	+	0	0	0
To. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
We. 1	0	0	0	0	0	0	0	0	0	0	0	+	0	0	0
Fe. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mc. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pi. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
" 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Te. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
" 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
An. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
" 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Wi. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ma. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
" 2	0	0	0	0	0	0	0	+	0	0	0	0	0	0	0
Syphilis															
A	0	0	0	0	0	0	0	0	0	0	0	0	0	+++	+++
B	0	0	0	0	++	0	0	0	+	0	0	0	0	0	0
C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D	0	0	0	++	++	0	0	0	+	0	0	0	0	0	0

* Serum 2 is at a later period in the course of the disease.

scopically visible. When the surface of the particles is coated with antigen, the whole particle exhibits many of the properties of its protein covering (31). A reaction of antigen on the particle surface with its antibody results in macroscopic agglutination (32).

Experimental.—Colloidal particles were prepared by the method of Cavelti (33). To tubes containing 0.2 ml. of a mixture of collodion particles and a dilution of a clear saline extract of

the antigen, 0.5 ml. of antiserum in various dilutions was added. After being mixed and standing at room temperature for 2 hours, the mixture was centrifuged, agitated, and the degree of agglutination was observed.

Tests were made to determine the optimal concentration of particles, antigen, and antibody, using two known systems: rat kidney and rabbit anti-rat kidney serum R 673 (obtained from Dr. B. C. Segal), and the type-specific polysaccharide SS II of the Type II pneumococcus (obtained from Dr. M. Heidelberger) and its homologous rabbit antibody. Although the collodion particle method appeared to give positive results with known antigen-antibody systems, inconstant quantitative relationships were obtained at various times when the same system was repeated. No false positive results were obtained with good preparations of collodion particles.

Five or more tissues were obtained within 12 to 24 hours after the death of three individuals. One case (H.) had inactive rheumatic heart disease and pulmonary edema. The other two cases had carcinoma of the gall bladder (M.) and chronic glomerulonephritis (W.). In addition, heart tissue from one active rheumatic individual, and tonsil specimens from two inactive rheumatic children were also obtained. The tissues were kept in the solid CO₂ storage chest until ready for use. They were then ground with sand and extracted with cold saline to make a 20 per cent mixture by weight. After centrifugation, the clear supernate was separated for use in the tests. In all instances the tissues were from individuals of blood group O. All the tests reported employed group O sera to obviate the possibility of a false positive test due to an incompatible blood group reaction. For control tests, normal sera and uncoated normal particles were used, as well as the variety of tissues and concentrations. In addition, a precaution not previously employed was used. Known positive Wassermann sera from syphilitics served as controls.

Several batches of particles occasionally gave a preponderance of negative or of positive results with the tissue extract-serum systems, although particles from the same lot appeared to be similar to previous batches in their reaction with the known antigen-antibody systems.

A summary of some of the tests employing the collodion particle method is seen in Table II. All the sera listed here are from active rheumatic or syphilitic patients. Normal sera rarely gave a positive reaction with one of the tissue extracts. The sera from ten of the active rheumatic patients gave no reaction, or a rare weakly positive agglutination. Five gave positive reactions, usually against more than one tissue. In other tests, not shown here, these and other sera appeared to cause most marked agglutination with extracts of tonsil, spleen, kidney, and lung. Since streptococci were cultured from the tonsils at operation, antibodies to the streptococci were probably instrumental in causing the marked agglutination of the tonsillar tissue.

Discussion.—The occurrence in rheumatic sera of agglutinins to collodion particles coated with a heart tissue extract was observed infrequently as compared with reactions to particles coated with extracts of other tissues. In addition, sera from syphilitic patients appeared to contain the agglutinins more frequently than did rheumatic sera. Since we performed these tests, Dr. Cavelti has informed us that he has had difficulty repeating his results with antigens other than the extract of the one heart tissue which gave strongly positive reactions. The diminution of his supply of the original antigen precludes any attempt at analysis. The possibility continues that the reaction noted

constitutes (1) a reaction between antibodies and bacterial contaminants of the tissues used, or (2) a modification of the flocculation tests for syphilis, since the Wassermann antigen is a constituent of normal tissues (34, 35). The occurrence of biologically false positive Wassermann reactions in many diseases has been reviewed by Davis (36).

SUMMARY AND CONCLUSIONS

1. An attempt was made to repeat and extend various tests which have been presumed to demonstrate specific antigens and antibodies in rheumatic fever.

2. The "phase reaction" appears to be an inconstant phenomenon probably related to a colloidal abnormality of the serum, rather than to a specific antigen-antibody system.

3. No specific autoantibodies to human tissue extracts were demonstrable by complement fixation or by the collodion particle technique. Variable results were noted with the same test sera on different occasions, and positive reactions with control tissues and control sera were observed.

4. The possibility should be considered that autoantibodies are not necessarily specific for rheumatic fever but may be manifestations of the occurrence of a type of reaction similar to a biologically false positive Wassermann reaction.

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THE CYTOLOGY OF RICKETTSIAE

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PLATES 35 AND 36

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In recent years it has been shown that all bacterial cells which have been adequately examined are essentially similar to the cells of higher organisms with the demonstration of desoxyribonucleic acid-containing, regularly dividing nuclear structures and the presence of ribonucleic acid in the cytoplasm (1, 2). It is not yet clear whether these Feulgen-positive bodies are similar to chromosomes in higher organisms or whether the genic material is organized in a different way. Since chromosomes exhibit a very special structure and behavior during cell division in addition to containing DNA and being self-reproducing, this name should not be applied to the Feulgen-positive bodies of bacteria. Instead the less specific terms "nuclear structure" and "chromatinic body" (Robinow (1)) will be used here.

Rickettsiae are usually considered to be essentially like bacteria in morphology though they resemble viruses in being obligate intracellular parasites (cf. reference 3). Photographs with the electron microscope have revealed some internal structures similar to those found in bacteria (4). Chemical analysis of isolated rickettsiae, however, has shown the presence of desoxyribonucleic acid only, no ribonucleic acid having been detected (5, 6). The present study was undertaken in order to investigate, first, whether RNA can be demonstrated in unwashed rickettsiae using cytochemical methods, and secondly, whether the DNA is present in nuclear structures as in the bacteria above mentioned, or is diffusely distributed through the rickettsial bodies.

Materials and Methods

The material used in this study came from chick embryo yolk sacs infected with the Breinl strain of epidemic typhus (*Rickettsia prowazekii*). Yolk sac smears were air-dried, heat-fixed, and then immersed in Carnoy or in 20 per cent formalin. Concentrated suspensions of rickettsiae were obtained from yolk sac emulsions by repeated washing in saline.

Ultraviolet photographs (2537 Å) were obtained using a G.E. germicidal lamp (4 watt) with quartz-condensing lens, a Bäckström filter (20 per cent NiSO₄ plus 8.5 per cent CoSO₄ in distilled water), Zeiss 1.7 mm. quartz objective and Zeiss ×10 quartz ocular.

Rickettsiae for electron microscope photographs were extracted from yolk sacs, sulfate-precipitated, and inactivated with 1:5,000 merthiolate. A drop of this suspension was dried on formvar film, washed in distilled water to remove salts, and dried again for examination in the RCA Universal electron microscope.

Unstained smears of rickettsiae were also photographed with the phase contrast microscope (Spencer 1.8 mm., medium dark contrast objective).

To determine the presence of RNA in unwashed rickettsiae they were fixed in 20 per cent formalin and treated with ribonuclease (preparation of Dr. Kunitz, 0.2 mg. per ml. in distilled water, 45 minutes at 50°C.). Controls were treated the same way except for the enzyme. Buffer solutions were not used because they were found to extract the basophilic material from rickettsiae on the control slides. The slides were then stained together in methyl green pyronine for 20 minutes and differentiated in acetone.

Demonstration of Ribonucleic Acid in the Cytoplasm of Rickettsiae

Tovarnickij *et al.* (5) studied the chemical composition of rickettsiae isolated from mouse lungs and washed with physiological saline. Cohen (6) analyzed rickettsiae isolated from phenol-treated typhus vaccines. Both authors reported the presence of DNA, but no RNA was found. They concluded that rickettsiae were similar to viruses in containing only one type of nucleic acid, while bacteria and higher organisms always have both RNA and DNA. However, it has been shown that ribonucleoproteins are easily extracted from cells with physiological saline (7). It is therefore possible that no RNA was present in purified rickettsiae because it had been washed out during preparation. The presence of RNA in cells can be demonstrated cytochemically using ribonuclease and basic dyes (8). We therefore treated yolk sac smears fixed with 20 per cent formalin with ribonuclease and stained with methyl green pyronine. On the control slide the rickettsiae stain more or less solidly red with pyronine (Fig. 1). The intensity of the staining varies somewhat from one cell to the other. After digestion with ribonuclease, however, the over-all staining is always very much decreased (Fig. 2). Rickettsiae therefore contain RNA in variable amounts, probably depending on the physiological state as has been demonstrated for bacteria (9). Since it was not found in purified suspensions of rickettsiae it must have been lost during preparation. The effect of saline for instance on the staining with pyronine is marked. Fresh rickettsiae and rickettsiae washed with saline were smeared on the same slide and stained with pyronine. Unwashed rickettsiae stain uniformly red. Rickettsiae washed once stain very faintly and those washed more thoroughly do not stain at all with pyronine.

Recently Callot and Vendrely (10) studied the effect of desoxyribonuclease and ribonuclease on rickettsiae. They found that after desoxyribonuclease the staining with Giemsa was greatly reduced, but no marked decrease in staining was detected after digestion with ribonuclease. It is possible that the RNA was washed out during incubation in the control, or that they were dealing with rickettsiae in a physiological state with low RNA content in the cytoplasm.

Demonstration of Nuclear Structures in Rickettsiae

With the phase contrast microscope two or more dark bodies are visible in the rickettsial rods (Fig. 3). These structures are very similar to the chroma-

tinic bodies in bacteria. In order to determine whether they are nuclear structures like those in bacteria it must be shown that they contain DNA.

(a) *Staining with Basic Dyes.*—In bacteria the nuclear structures can be demonstrated with basic dyes after removal of the RNA of the cytoplasm. This is accomplished either with ribonuclease (11) or through hydrolysis with 1 N HCl (12). Robinow (13) hydrolyzes in 1 N HCl and then stains with Giemsa.

Rickettsiae in fresh yolk sac smears stain solidly with basic dyes such as basic fuchsin (Macchiavello's procedure) and pyronine. Rickettsiae which have been washed with saline before fixation lose the ability to stain with these dyes. If washed rickettsiae, or rickettsiae hydrolyzed with 1 N HCl at 60° for 10 minutes are stained with Giemsa chromatinic bodies become apparent (Fig. 4).

Methyl green is a basic dye with high specificity for DNA. Washed rickettsiae were stained with methyl green pyronine. The nuclear structures stained purplish and the cytoplasm faintly pink. Photographed at 630 m μ near the absorption maximum of methyl green, the nuclear structures were clearly visible. The chromatinic bodies, however, appeared most distinct after treatment with ribonuclease and staining with basic dyes. Fig. 2 shows rickettsiae stained with methyl green pyronine after ribonuclease treatment. The nuclear structures stained purplish and stand out clearly in the practically colorless cytoplasm. Fig. 6 is a photograph from the same slide, but taken with the phase contrast microscope.

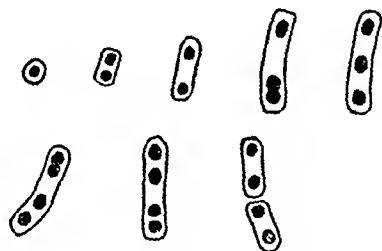
(b) *Ultraviolet Absorption.*—Photographs of washed rickettsiae at 2537 Å show strongly absorbing structures inside the rickettsial bodies (Fig. 5), corresponding to the structures staining with basic dyes. This is further evidence for the presence of nucleic acid in these structures.

(c) *Feulgen Reaction.*—Yolk sac smears were fixed in Carnoy and stained with the Feulgen reaction (modification of Rafalko (14)). The nuclear structures stained very faintly red. With a green filter (Wratten 74) the small dots of the chromatinic bodies could be seen, but nothing else of the rickettsiae was visible. Though the stain was so weak that by itself it would be questionable as a demonstration of DNA, it indicated that the DNA found in purified rickettsiae must be concentrated in these small structures inside the rickettsial bodies. The absolute amount of DNA in one rickettsial organism was obviously extremely small.

The behavior of these chromatinic bodies towards basic dyes, especially after digestion with ribonuclease, the absorption at 2537 Å, and the Feulgen staining therefore leave little doubt that the DNA found in rickettsiae is localized in definite nuclear structures. Spherical rickettsiae contain one nuclear body. In rod-shaped rickettsiae one finds two bodies which are close together in short rods and farther separated in long rods. Sometimes long rods may contain

three or four chromatinic bodies. These are usually spherical, but occasionally one sees dumbbell-shaped structures which suggest a chromatinic body in the process of division (Text-fig. 1, and Figs. 6 and 9).

Electron microscope photographs of rickettsiae washed with saline revealed internal structures which correspond to the chromatinic bodies described above.¹ Rickettsiae with one, two, or three chromatinic bodies were common (Figs. 7 to 12). Sometimes two nuclear structures were very close together, possibly representing the division of a chromatinic body (Figs. 9 and 10). Plotz *et al.* (4) described structures which seem to be identical with our chromatinic bodies.



TEXT-FIG. 1. Nuclear structures in various forms of *Rickettsia prowazeki*. Compare with Figs. 6 and 7 to 12.

SUMMARY

Internal structures of rickettsiae seen with phase contrast microscopy and in the electron microscope contain desoxyribonucleic acid and are therefore nuclear structures similar to those found in bacteria. They are minute spherical bodies, either single as in spherical rickettsiae or varying in number from 2 to 4 in rod-shaped forms. Occasional dumbbell-shaped chromatinic bodies are thought to represent these structures in the process of division. The presence of ribonucleic acid in the cytoplasm of rickettsiae was demonstrated with the use of ribonuclease and basic dyes. Rickettsiae therefore have a cellular organization similar to that of certain bacteria, with a clear differentiation into nuclear structure and cytoplasm.

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¹ The electron microscope photographs were prepared by Dr. E. G. Pickels, formerly at the Rockefeller Institute.

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EXPLANATION OF PLATES

PLATE 35

FIGS. 1 and 2. Rickettsiae in yolk sac smears, stained with methyl green pyronine. Fig. 2 shows the rickettsiae after treatment with ribonuclease, Fig. 1 in the control slide. In the control the cytoplasm is stained intensely with pyronine. After ribonuclease treatment only the nuclear structures are stained. Zeiss 2 mm. NA 1.3 objective, $\times 2400$.

FIG. 3. Photograph of unwashed rickettsiae in yolk sac smear taken with the phase contrast microscope. Spencer 1.8 mm. dark medium, $\times 2700$.

FIG. 4. Rickettsiae in yolk sac smear, hydrolyzed with N HCl 10 minutes, stained with Giemsa. Zeiss 2 mm. NA 1.3 objective, $\times 2400$.

FIG. 5. Rickettsiae washed with saline, photographed in ultraviolet light (2537 Å), Zeiss 1.7 mm. quartz objective, *ca.* $\times 2500$. The nuclear structures absorb more intensely than the cytoplasm.

FIG. 6. Rickettsiae in yolk sac smear, digested with ribonuclease, stained with methyl green pyronine, photographed with the phase contrast microscope. Spencer 1.8 mm. dark medium objective, $\times 2700$. The chromatinic bodies stand out most sharply with this technique.

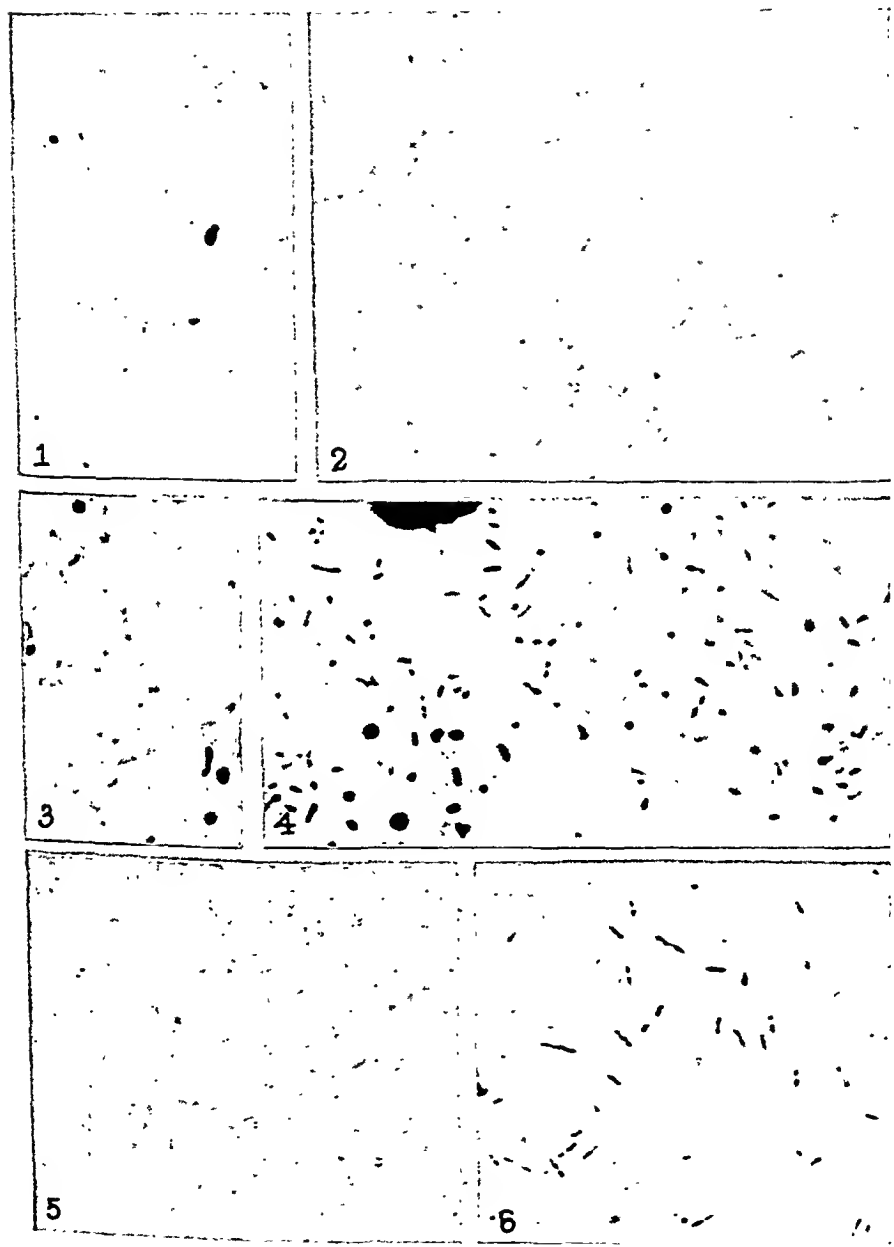
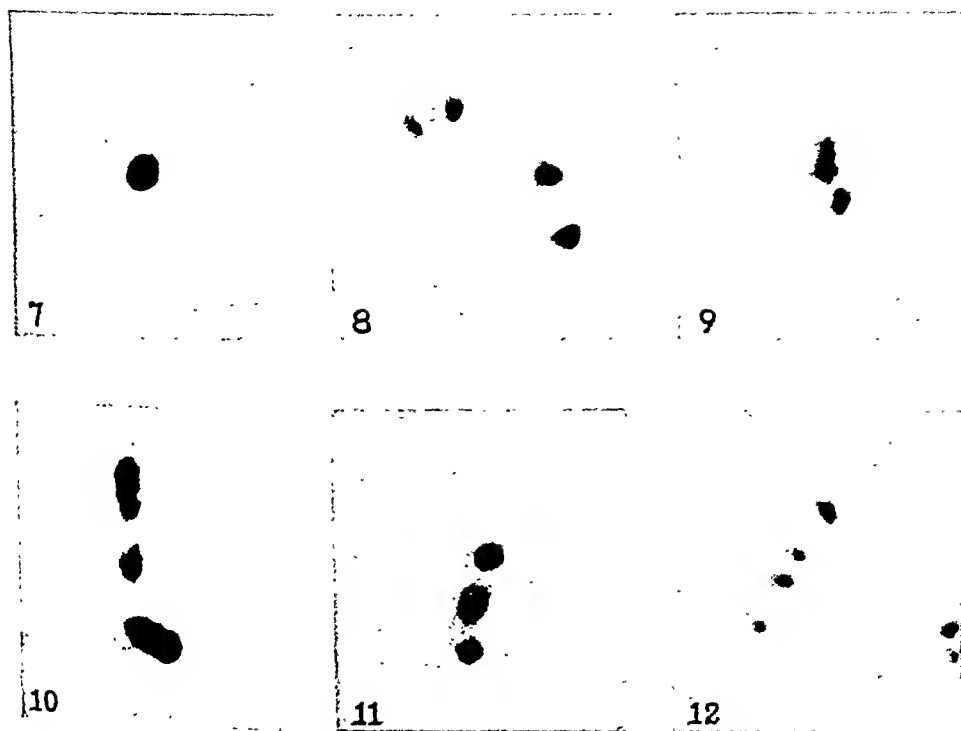


PLATE 36

FIGS. 7 to 12. Electron microscope photographs of rickettsiae extracted from yolk sacs, sulfate-precipitated, and inactivated with merthiolate. RCA Universal electron microscope, *ca.* $\times 15,000$. Compare with Fig. 6.



INDUCTION OF CARDIAC LESIONS, CLOSELY RESEMBLING THOSE OF RHEUMATIC FEVER, IN RABBITS FOLLOWING REPEATED SKIN INFECTIONS WITH GROUP A STREPTOCOCCI

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PLATES 37 TO 42

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Elucidation of the rôle of group A streptococci in the pathogenesis of rheumatic fever might be furthered if a host alteration closely simulating this disease could be induced in laboratory animals infected with these microorganisms; but to date efforts in this direction have failed. This failure possibly stems from one or more factors: (1) lower animals may be incapable of developing the disease; (2) the streptococci employed may have been unable to induce the characteristic host alterations; (3) the experimental conditions may have been unsuitable.

Because a spontaneous disease closely resembling rheumatic fever has not been found in lower animals, its experimental induction might be impossible. The streptococci usually pathogenic for animals belong to serological groups other than A, whereas group A streptococci are chiefly pathogenic for man, and in so far as is known this group comprises the only streptococci that induce the respiratory infections preceding rheumatic fever; the host-parasite relationships among lower animals and streptococci may not be reflected in a rheumatic fever-like state. In experimental streptococcal infections, single strains have usually been employed; but valid data indicate that successive group A streptococcal infections in one person are probably caused by different serological types (1, 2). Rheumatic fever, moreover, occurs among patients in an age period and under conditions which make it probable that they had experienced one or more previous streptococcal infections.

In investigating possible relationships between rheumatic fever and various states of altered reactivity induced experimentally in animals infected with streptococci, workers in this laboratory observed the following phenomena: (a) focal infections of rabbits with viridans, group A or C streptococci resulted in the development of clear cut cutaneous and general hyperreactivity to the homologous infecting strain (3, 4), which was markedly enhanced by frequently repeated minute intracutaneous inoculations (5); (b) intravenous injections of living viridans streptococci (6), or of heat-killed vaccines of group A or C streptococci (5) induced in rabbits a state of diminished cutaneous reactivity

* This work was done during the tenure of a Life Insurance Medical Research Fellowship.

to inoculation with homologous strains; (c) these immunized animals simultaneously showed cutaneous hyperreactivity to strains belonging to heterologous groups (4, 7); (d) some rabbits immunized intravenously or subcutaneously with heat-killed vaccines of one type of group A streptococci developed decreased skin reactivity to intracutaneous inocula of the same type, but simultaneously showed greater than normal cutaneous reactivity to minute intracutaneous inocula with heterologous types, and the same often held true when the preliminary immunization was induced by repeated skin infections with a strain of a very rabbit-virulent group A streptococcus (8), and the state of cutaneous hyperreactivity was brought out far more clearly with high dilutions of the challenging inocula than with low dilutions; (e) rabbits resting 2 or more months from immunization with viridans, group A or C streptococci became cutaneously and systemically hyperreactive to the homologous strain of streptococci previously injected (7). The following information was also available: Rheumatic fever patients develop type-specific antibodies to the group A streptococcus inducing the nasopharyngeal infection preceding a rheumatic fever attack (1, 2); rheumatic fever patients, as a rule, were found to be hyperreactive to viridans and group A streptococcal nucleoproteins (9) and to group A streptococcal vaccines (10). It was later assumed that this hyperreactive state was induced by recurring focal (nasopharyngeal) infections with a succession of different serological types of group A streptococci.

These observations suggested that a rheumatic fever-like state might be induced in animals by successive focal group A streptococcal infections, each caused by a serological type heterologous to those previously employed. This communication reports the first testing of this hypothesis.

Methods

Because it was desirable to test a relatively large group of animals, and as considerable information was available concerning the reactivity of rabbits to streptococcal infections, this species was chosen. New Zealand Reds and a cross-breed designated hare brown, all bred in the Rockefeller Institute, were usually employed; occasionally chinchilla and other varieties were tested. All rabbits were fed approximately 560 gm. of Rockland rabbit ration pellets within each week. Animals with skins which mostly remained bare for considerable periods after close clipping were preferred; and such clipped sites were used primarily; but after repeated inoculations it sometimes became difficult to find very suitable skin, and coarsely hairy areas had to be inoculated.

The group A streptococci employed all exhibited matt or mucoid colony forms in 18 to 24 hours growth on moist rabbit blood agar; and were shown to produce large amounts of type-specific M protein in Todd-Hewitt broth made with neopeptone. Mostly they had only moderate virulence for rabbits, but even in this respect there was considerable variation, both among the various types employed and in different subcultures of single strains. Efforts to increase virulence by rabbit passage have been only moderately successful.

Sixteen to 20 hour Todd-Hewitt neopeptone broth cultures were serially diluted in tenfold steps with broth, and the inocula in 0.1 cc. volume, containing between 10^{-8} and 10^{-2} cc. of the original culture, were injected into closely clipped skin of right and left gluteal, lumbar, thoracic, or shoulder areas. Ten times more cocci were injected on the right side than on the

eft. In the original groups of animals, the 2nd to 4th successive focal cutaneous infections are set up in the same well healed, but scarred, gluteal sites. Subsequently, because of re-

TABLE I
Rabbit 70-58—New Zealand ♀

Infections					Course of infections				
Date	Streptococcus type	Inocula	Skin site	Skin reaction	Date	Weight	ESR	ASO units	Remarks
1945					1945	kg.	mm./hr.		
7/17	1	10 ⁻⁸	Gl	N	7/17	3.0			
8/28	11	10 ⁻⁸	Gl		8/19	3.1			
10/8	17	10 ⁻⁸	Gl		10/30		1		
11/12	13	10 ⁻⁴	Gl	Initially >N; <N in few days	11/12	3.8		<25	
11/15	13	10 ⁻⁴	Gl		11/16		1		
11/19	13	10 ⁻³	Gl		11/25		1	25	
11/25	13	10 ⁻³	Gl		11/28		1	25	
1947					1947				
1/13	3	10 ⁻⁴ , 10 ⁻⁸	Gl;	Th—>N	1/10	3.7			
					1/18		1	25	
					1/20	3.8			
4/18	19	10 ⁻⁴ , 10 ⁻⁸	Gl;	Sh—>N	4/16	4.0			
					4/25		1	50	
5/28	17	10 ⁻³ , 10 ⁻⁸	Gl;	Lu—<N	5/27			25	
					6/7		1	25	
9/16	1	10 ⁻⁴ , 10 ⁻⁸	Gl;	Th—>>N	9/19				*
					9/22				Anorexia
					9/25		130	400	"
					9/26		47	>1000	"
					9/27		76		" ‡
					9/29				Died§

N, average cutaneous response of normal control rabbits to intracutaneous inoculation with streptococci.

Gl indicates gluteal; Lu, lumbar; Th, thoracic; Sh, shoulder.

* Erythema over right knee.

† Negative blood culture.

§ Autopsy blood culture negative.

E.S.R. of normal rabbits, 1 to 2 mm. per 1 hour and 2 to 4 mm. per 2 hours.

ESR, erythrocyte sedimentation rate (Westergren).

ASO, antistreptolysin O titer.

sults recorded below, each reinfection, usually with a type of streptococcus not previously injected, included 4 areas: right and left scarred gluteal skin sites and a right and left skin site least likely to have been locally inflamed in previous infections.

All skin lesions were measured daily until recession was demonstrated. The condition of the rabbits was observed; and their weights were recorded at suitable intervals. Blood was obtained from the ear veins for serum which was refrigerated and later tested for antistreptolysin O and antistreptokinase content, and for precipitin reactions with extracts of homologous and heterologous types of group A streptococci. These data furnished a rough index of the serological responses to the infections. During the earlier experiments erythrocyte sedimentation rate determinations (Westergren method) were made once or twice during the

TABLE II
Rabbit 71-77—Hare Brown ♂

Infections					Course of infections					
Date	Streptococcus type	Inocula	Skin site	Skin reaction	Date	Weight	ESR	WBC 1000	ASO units	Remarks
1947					1947	kg.	mm./hr.			
5/ 8	19	10^{-4} , 10^{-4}	Gl;	Sh—>2 other norms	5/8	2.7				
6/20	1	10^{-4} , 10^{-5}	Gl;	Lu—<<N	6/20				<25	
9/16	1	10^{-4} , 10^{-5}	Gl;	Th—<<N						
11/ 6	London	10^{-3} , 10^{-4}	Gl;	Th—=N	11/ 4				<25	
					11/19		3		75	
1948					1948					
1/16					1/16		1	7.7	25	
2/ 6	3	10^{-3} , 10^{-4}	Gl;	Th—<N	2/ 6	3.4				
					2/14	2.8	152	10.8	600	Anorexia, marked, cardiac irregularity* died*

* Negative blood culture ante- and postmortem.

fortnight following the inoculations; but later both erythrocyte sedimentation rate determinations and leucocyte counts were made twice or thrice weekly until they were approximately normal or until the animal died or was sacrificed.

Where indicated, blood cultures were made from living rabbits with blood obtained from ear veins and placed both in Todd-Hewitt neopeptone blood broth and on rabbit blood agar plates. Blood obtained postmortem from the inferior vena cava of all dead rabbits was similarly cultured; and streptococci recovered were identified serologically.

During the first 4 to 5 months' experimentation it was found that successive monthly to bimonthly inoculations with streptococci of different serological types into the same gluteal skin sites usually induced progressively smaller local lesions than those which followed similar inoculation in comparable skin of normal controls; but in their previously uninfected (e.g.

thoracic) skin the same sized inoculum almost invariably induced greater local inflammation than in their multiply infected gluteal skin or in the thoracic skin of normal controls (Table I;

TABLE III
Rabbit 71-80—Hare Brown ♀

Infections				Course of infections							
Date	Streptococcus type	Inocula	Skin site	Skin reaction	Date	Weight kg.	ESR		WBC 1000	ASO units	Remarks
							1 hr.	2 hr.			
1947					1947						
5/28	6	10 ⁻² , 10 ⁻⁴	Gl; Lu	>2 other norms	5/28	3.4					
					6/7	2.7				700	
6/20	1	10 ⁻⁴ , 10 ⁻⁵	Gl; Th	<N	6/20	2.8				<25	
9/16	1	10 ⁻⁴ , 10 ⁻⁵	Gl; Th	<N	9/30		1			100	
11/6	London	10 ⁻² , 10 ⁻⁴	Gl; Sh	=N	11/3					100	
					11/14	3.3					*
					11/18	3.2	1			75	†
1948					1948						
2/6	3	10 ⁻² , 10 ⁻⁴	Gl; Th	<N	1/19	3.4	1	16.2		25	
					2/16	3.4	4	19.3		50	
					2/19		2	14.2		75	
					2/25		3	13.5		25	
9/21	9	10 ⁻² , 10 ⁻⁴	Gl; Th	=N	5/26	3.5					
9/22	9	10 ⁻² , 10 ⁻²	Lu		9/21	3.5	1	18.0			
					9/22			22.5			
					9/23		5 11			<25	
					9/24	3.1	7 16	18.9		<25	
					9/26	2.9					
					9/27	2.7	29 56	30.1		50	
					9/28	2.7	33 54	21.8		50	Anorexia
					9/29		49 80			75	Diarrhea
					10/1	2.3	45 88	19.5		200	
					10/2	2.4				100	Sacrificed§

* Guards hind legs.

† Negative blood culture.

§ Autopsy blood culture negative.

January, 1947). It was, therefore, obvious that new areas were requisite to obtain a rough approximation of an animal's cutaneous reactivity to successive inoculations. Furthermore, because of this finding that succeeding streptococcal skin inflammation was likely to be more

intense in previously uninflamed skin than in scarred sites, it seemed possible that streptococci might survive longer and effect more sustained local infection in fresh skin than in scarred areas; and that from the larger inflammatory zones more toxic material might be elaborated and absorbed than from the small lesions.

Variations in the inoculation procedures are illustrated in Tables I, II, and III. Occasionally, as in November, 1946 (Table I), or in September, 1948 (Table III), repeated intracutaneous inoculations with the same type of streptococcus were given within a few days in attempts to enhance the infectious stimulus of lowly virulent strains. In other instances, as with type 1 (Tables I, II, and III), the same type was reinjected after a relatively long interval; but generally each successive inoculation in a given animal was with a type heterologous to those previously used to infect a given animal.

Autopsies were performed as soon after death as possible; those on sacrificed rabbits were carried out immediately after exitus, usually effected with intravenous sodium nembutal. Tissues were fixed in Zenker-acetic acid, and sections cut from paraffin blocks were stained with hematoxylin and eosin, Giemsa, Weigert-hematoxylin and eosin, Masson's trichrome, Mallory's aniline blue, and where indicated with Gram-Weigert and malachite green-acridine red (11), a technique applicable to Zenker-fixed tissues, whereas the Unna-Pappenheim methyl green-pyronine technique requires alcohol fixation.

RESULTS

After sustaining 2 to 10 infections with streptococci of different serological types within 3 to 20 months, some rabbits sickened and showed various combinations of the following signs and symptoms: elevated erythrocyte sedimentation rates for 1 to 2 weeks; leucocytosis; anorexia; weight loss; postexertional dyspnea; occasional transient pulmonary rales; tachycardia; and in a few instances, definitely irregular cardiac rhythm. Many of these rabbits recovered; a portion were sacrificed within 10 to 14 days following their last infection while exhibiting definite symptoms, leucocytosis, and elevated erythrocyte sedimentation rates higher than were occurring in normal controls; in several rabbits, however, a severe illness developed following the last streptococcal infection and terminated fatally, whereas some of the normal controls survived the same streptococcal infection. A few in the fatal group died within 2 to 5 days following the last infection (even though normal controls in some instances survived) and in all except one of these rapidly fatal cases, streptococcal bacteremia was established at autopsy. In about half of a group of rabbits dying spontaneously between 6 and 14 days after the last infection, streptococcal bacteremia was demonstrated at autopsy; in the other rabbits of this group, however, streptococci could not be cultured from the blood either before death or at autopsy.

In the hearts of the successively infected rabbits which had sickened and succumbed, and of those sacrificed while sick, there have been found on microscopic examination focal alterations in the connective tissue framework in blood vessel adventitia, valves, mural endocardium, epicardium, and in the myocardial interstitium. Many collagen fibers in these sites are swollen; some are intensely eosinophilic, others stain poorly; some swollen collagen fibers stain entirely, whereas others stain in patchy fashion like fibrin with both Masson's trichrome and Mallory's connective tissue techniques. Arranged about and interspersed

in fields of swollen "fibrinoid" collagen are nodular collections of large, irregularly shaped cells, often with abundant, finely granular basophilic cytoplasm which takes a smudgy red color with the malachite green-acridine red stain. Often these cells have very indistinct outlines; some have long streamer-like cytoplasmic processes which gradually fade into the contiguous areas. The vesicular nuclei, single or multiple, are variously shaped, and have sharply defined membranes. Clumping of chromatin often leaves the rest of the nucleus clear. Some nuclei are pyknotic. Cells with multiple, centrally placed nuclei, 2 to 10 in number, occur in greatest profusion in the mitral and aortic sulci and rings and in the endocardium (Fig. 3). The lesions also contain many cells of the Anitschkow myocyte type, and occasionally small round cells and polymorphonuclear leucocytes, both pseudo- and true eosinophiles. The sites of predilection for the occurrence of these nodular granulomata in most hearts are endocardial, subendocardial, and blood vessel adventitia and paraadventitia. These adventitial lesions are by no means limited to the roots of the valves, but at times are conspicuously present throughout the hearts, particularly in the left ventricle and interventricular septum.

In some hearts the granulomata occur in the loose myocardial interstitium unassociated with arteries or veins but, in most instances, with capillaries. In agreement with Gross (12) the latter are designated "myocardial granulomata" to distinguish them from granulomata associated with other cardiac structures. Interstitial valvulitis, most marked in the middle of the cusps, has occurred commonly in the mitral and aortic valves and also in the right auriculoventricular valve; these areas beneath the line of closure show edema of varying intensity and cellular components like those in the submiliary nodules. Marked proliferation of mitral and aortic valvular endocardial and subendocardial cells occurs in several hearts to create many layered palisades containing numerous multinucleated giant cells dispersed in swollen or "fibrinoid" collagen (Figs. 1 and 2). These lesions are occasionally limited to the sulci, but are also found often on both surfaces of the valve and of the chordae tendineae (Fig. 10). At times the most superficially palisaded cells have apparently undergone necrosis and conversion into acellular material that stains like fibrin. The latter phenomenon was most marked in rabbits dying spontaneously within 2 weeks after the final infection. On no valves were there seen, macroscopically, rows of fine verrucae along the lines of closure. In the gross, however, the mitral valve of several rabbits showed along the line of closure a row of fine discrete opalescent elevations usually more marked on the aortic leaflet. Microscopically these elevations consist of interstitial edema and valvulitis which in some instances are more intense than in the neighboring tissues. Occasionally larger fine, firm white nodules projecting from the surface of the valve were visible. Foci of frankly "fibrinoid" collagen¹ are seen in auricular (Fig. 12) and ventricu-

¹ The expression "frankly fibrinoid collagen" indicates that the altered collagen stains unequivocally like fibrin with Mallory's connective tissue and Masson's trichrome techniques.

lar epicardium in association with proliferated epicardial and subepicardial elements. These patches of epicarditis are microscopic in size; and no extensive plastic pericardial exudate has been detected in the gross.

Granulomata in the compact paravascular connective tissue differ in architectural configuration from the "myocardial granulomata" in the looser tissue between muscle bundles. There are submiliary granulomata closely resembling the coronal (Fig. 6), reticular (Figs. 4 and 7), and mosaic (Figs. 8, 9, and 11) types of Aschoff bodies described by Gross in human rheumatic hearts (12); and in the left ventricle and interventricular septum of a few rabbit hearts several myocardial granulomata are often seen in a low power field; but in no rabbit dying spontaneously or sacrificed within 2 weeks after final infection, have there been found well developed polarized or fibrillar types of granulomata. Gross considered the peculiarly shaped and arranged cells in such Aschoff bodies to represent terminal metamorphosis of the rheumatic granuloma cells into fibroblasts. Damage to myocardium adjacent to granulomata has been prominent, and has ranged from swelling and vacuolation of the myofibers and vesiculation of their nuclei to complete dissolution and replacement by scar. Occasionally apparent fusion of neighboring granulomata combined with extensive adjacent myocardial destruction and connective tissue replacement has resulted in macroscopically visible lesions in stained sections of left ventricle and papillary muscles.²

The coronary arterial system is variously altered. Fairly commonly there is marked intimal hyperplasia and elastification involving chiefly small arteries and arterioles. A well developed intimal musculoelastic hyperplastic lesion occurs in several rabbits. In the hearts of two rabbits there is found marked ramification of fibrinoid material throughout or surrounding the wall of a small artery or capillary (Fig. 5). Interspersed in and arranged about the extension of this intensely eosinophilic material into the tissue adjacent to the vessel are granuloma cells of the type found in the previously described rabbit granulomata. This vascular lesion closely resembles that described in rheumatic human hearts by Pappenheimer and Von Glahn (13). Panarteritis of the so called "allergic" or periarteritis nodosa type is conspicuously absent in the hearts of all intracutaneously infected rabbits. Verrucous and polypoid endarteritis are occasionally present. In the intima and immediately subadjacent media of the aorta near its root there occasionally is seen a lesion comparable with that in the valve sulci and closely resembling that described by Pappen-

² A striking increase in size of the adrenal glands occurred in rabbits dying, or sacrificed while sick, following the last of several intracutaneous streptococcal infections. Microscopically hyperplasia, hypertrophy, and necrosis of fascicular zone cells are seen. There is striking correlation between the degree of macroscopic enlargement of the fascicular zone of the adrenal cortex and the occurrence of myocardial granulomata. Detailed data concerning these observations will be presented shortly.

heimer and Von Glahn (13) in human rheumatic aortitis. Occasionally there are foci of clearly defined fibrinoid collagen in the adventitia of the root and first portion of the aorta. These lesions will be illustrated later.

Neither bacteria nor any structures resembling inclusion bodies have been seen in the above described lesions stained according to Gram-Weigert or Giemsa techniques. There has, moreover, been no calcification of the myocar-

TABLE IV

Rabbit groups	No.	Bacteremia	Acute rheumatic fever-like cardiac lesions	Myocardial scars or healed arteri- tis of rheu- matic type
1. Normal rabbits.....	8	—	0	0
2. I.v. vaccine (group A or C streptococci)....	8	—	0	0
3. Dying 1-18 days after 1 i.v. infection.....	20	20	0*	0
4. Sacrificed 1 and 4 mos. after 1 i.v. infection..	2	0	0	0
5. Sacrificed within 1 mo. after 1 i.c. infection..	4	0	0	0
6. Dying within 2 wks. after 1 i.c. infection....	13	12	0	0
7. Dying 3 wks. after 1 i.c. infection.....	1	1	1‡	0
8. Sacrificed 10 to 21 days after last of several i.c. infections..	37	0	7	8
9. Dying 2 to 5 days after 2nd i.c. infection....	3	2	3§	0
10. Dying 8 to 14 days after last of 5 to 9 i.c. infections.....	3	0	3	2
11. Dying 5 to 9 days after last of 2 to 9 i.c. infections.....	7	7	6	3
12. Dying several weeks after last of several i.c. infections.....	4	0	0	3
Totals.....	110	42	20	16

i.v., intravenous. i.c., intracutaneous.

* The hearts of two rabbits dying 12 and 18 days, respectively, after one i.v. inoculation show an acute exudative and necrotizing arteritis.

‡ Interstitial valvulitis marked; vascular adventitial and interstitial foci of lymphocytes and plasma cells and occasional foci of young mesenchymal cells in adventitia without demonstrable alteration of collagen; no necrotizing arteritis.

§ Slight interstitial valvulitis only.

dial lesions, a very conspicuous phenomenon in experimental myocarditis induced with filterable viruses.

Controls.—The tissues of rabbits of the same stock and breeds, both normal and subjected to various experimental procedures have been examined at intervals during the investigative period in order to learn whether comparable lesions were occurring in such control animals, for it should be recalled that a peculiar myocarditis was described by Loewe and his coworkers (14) among stocks of rabbits injected with various materials as well as among uninoculated

controls. These workers ascribed these lesions to a spontaneous epidemic in their stock. As indicated in Table IV, enough controls have been examined to eliminate fairly certainly the possibility that such an epidemic existed among the animals we used. The small focal lesions described by Miller (15) in rabbits' hearts have not been encountered among our present stock of experimental animals or controls; hence it seems probable that the cardiac lesions that developed in our animals bear no relationship to previously described spontaneous rabbit myocarditis.

Inspection of Table IV indicates that the cardiac lesions forming the basis for this report have developed only in rabbits that had undergone multiple, successive cutaneous infections with group A streptococci of different types. In most of the animals showing these lesions, ante- and postmortem blood cultures were negative, and with bacterial stains no bacterial cells could be seen in the lesions; hence it seems improbable that the fresh tissue alterations were due to a direct action of streptococcal cells at the site of injury. In some of the animals, there was evidence of terminal streptococcemia, but even so those animals dying acutely with streptococcemia following their first cutaneous infection, or from intravenous inoculation with streptococci have not developed these submiliary granulomata. Similar negative results were found in rabbits repeatedly immunized intravenously with heat-killed group A or C streptococcal vaccines, as well as in those sacrificed after one intracutaneous inoculation. It seems, therefore, that those finally dying with bacteremia following the last of multiple skin infections developed these cardiac lesions (in which repeated bacterial stains have been negative) because the final insult affected tissues peculiarly conditioned by previous focal infections. It seems quite possible that in this group of rabbits, the bacteremia was, in fact, a terminal event.

DISCUSSION

The cardiac granulomata described, which in many respects bear such a striking histopathological similarity to those of human rheumatic fever, have been encountered only in animals that had undergone multiple, successive cutaneous infections with group A streptococci of several different types. It, therefore, seems probable that the relatively long experimental period and the reconditioning that the animals' tissues underwent as a result of several focal infections with different types of group A streptococci were important factors in the pathogenesis of these lesions. In certain respects this experimental procedure follows the pattern encountered in rheumatic fever patients: they have successive infections with different types of group A streptococci, and these infections are usually focalized in the upper respiratory tract and accessory tissues. Because it was impractical to infect rabbits' throats and sinuses repeatedly, and because successive focal infections appeared hypothetically to be important, the rabbits' skin was selected for the repeated insults.

The carditis developed, moreover, following infections with the same microorganisms that have been repeatedly proven to occur in the infections that precede attacks of rheumatic fever in man. This unique sequential relationship could not be demonstrated until Lancefield's system of classification of streptococci was available (16). In rabbits made hyperreactive to viridans, group A and group C streptococci by repeated focal infections and then shocked with intravenous inoculations of homologous streptococci, cardiac lesions of this type were not encountered (17).

It seems expedient to compare the carditis herein described in rabbits with that in animals of the same species with serum disease or subjected to repeated parenteral injections of foreign protein. This will be the subject of a later communication; but available evidence seems to indicate that the over-all histopathological picture in the rabbits repeatedly infected with streptococci bears closer resemblance to that of human rheumatic carditis than does experimental serum disease carditis. The fatal termination within 6 to 14 days, of an illness developing after the last of several focal infections is a phenomenon which, to our knowledge, has not been recorded in rabbits repeatedly injected and shocked with foreign protein.

Among the random samples of rabbits subjected to the described experimental procedure, only a small portion have developed these cardiac lesions. It seems pertinent to mention that only a small proportion of human beings in this geographical area develop rheumatic heart disease, and today an even smaller proportion develop polyarthritis rheumatica. Among subjects who have recovered from previous attacks of rheumatic fever and in rheumatic families, the incidence is considerably higher. There has been no attempt to select specially susceptible stock among the animals used in these experiments.

On the basis of evidence derived from the experiments here reported and from studies of rheumatic fever in man, it seems justified to assume that similar host-streptococcus relationships may be operative and requisite in the pathogenesis of these cardiac lesions in rabbits and rheumatic carditis in man.

SUMMARY

Cardiac lesions, closely resembling those found in rheumatic fever, have developed in rabbits that sickened following multiple, successive skin infections with several serological types of group A streptococci.

It is a pleasure to acknowledge the valuable technical assistance of Miss Jeanne Epstein and Mr. Andrew Littell.

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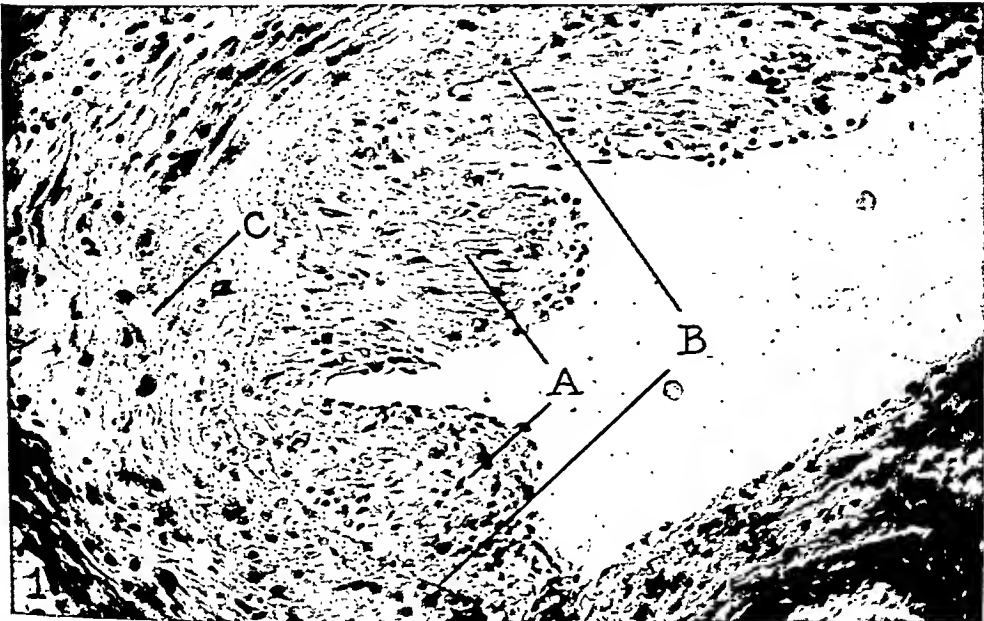
EXPLANATION OF PLATES

The photographs were made by Mr. Julian Carlile and Mr. Richard Carter.

PLATE 37

FIG. 1. Rabbit 73-13, sacrificed 15 days after last of 4 infections; no bacteremia at autopsy. *A*, polypoid endo- and subendocardial proliferation (palisade) in mitral sulcus; *B*, external elastic lamella; *C*, focus of frankly fibrinoid collagen. Weigert-hematoxylin and eosin. $\times 195$.

FIG. 2. Rabbit 71-77 (see Table II),—died 8 days after last of 5 infections; no bacteremia ante- or postmortem. *A*, extensive endo- and subendocardial proliferation (palisade) in aortic pocket; *B*, inflammation in annulus; *C*, aortic interstitial valvulitis; *D*, root of aorta. Weigert-hematoxylin and eosin. $\times 116$.



(Murphy and Swift: Induction of cardiac lesions)

PLATE 38

FIG. 3. Rabbit 71-77,—higher magnification of *A*, Fig. 2; numerous mono- and multinucleated cells, some with bizarre shaped nuclei, basophilic cytoplasm, and indistinct cytoplasmic outline; *E* and *F*, cells with 8 nuclei. Hematoxylin and eosin. $\times 886$.

FIG. 4. Rabbit 71-77,—reticular myocardial granuloma, interventricular septum. *A*, swollen collagen fibers forming interlacing network; collagen framework which assumes a direction roughly parallel with the myocardial bundles; *B*, cell with abundant cytoplasm; necrosis of adjacent myofibers. Weigert-hematoxylin and eosin. $\times 395$.

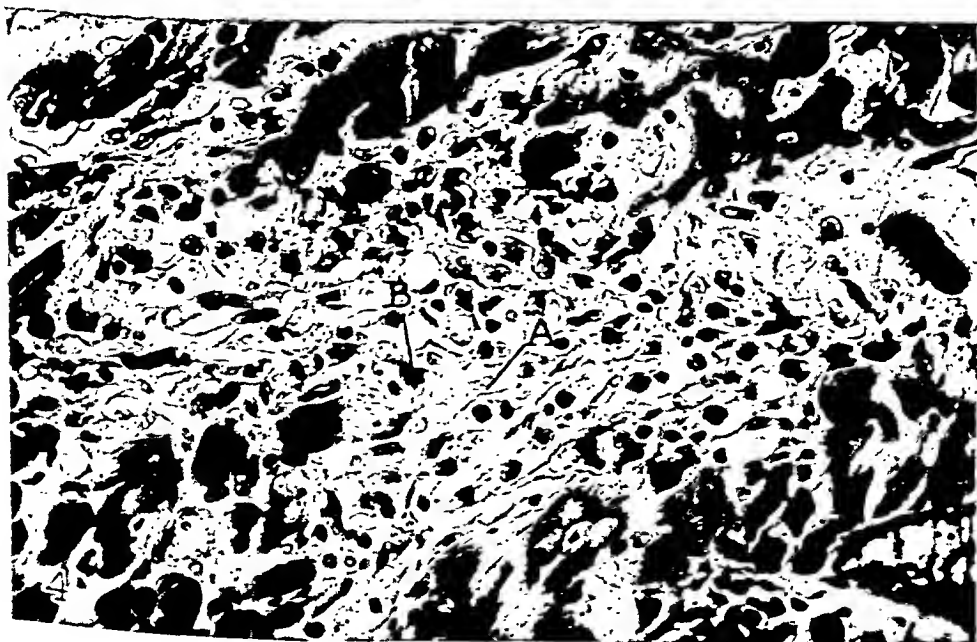
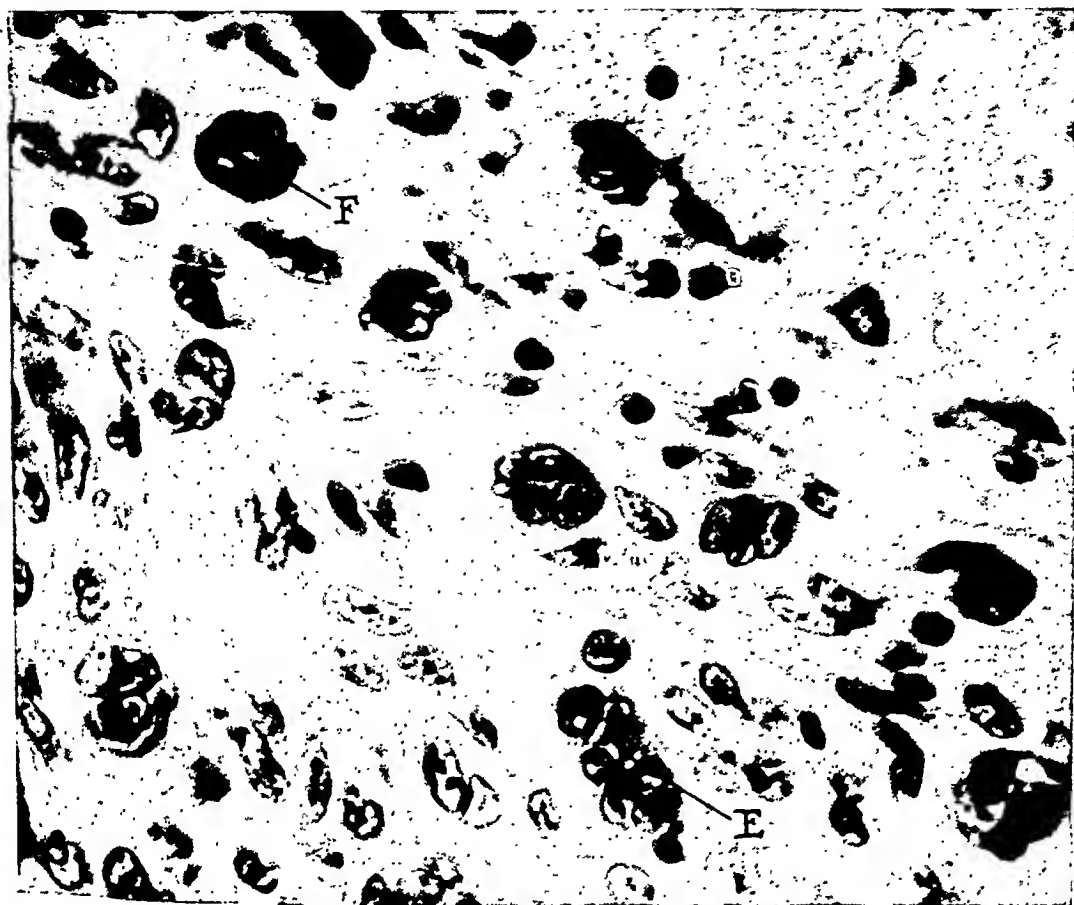
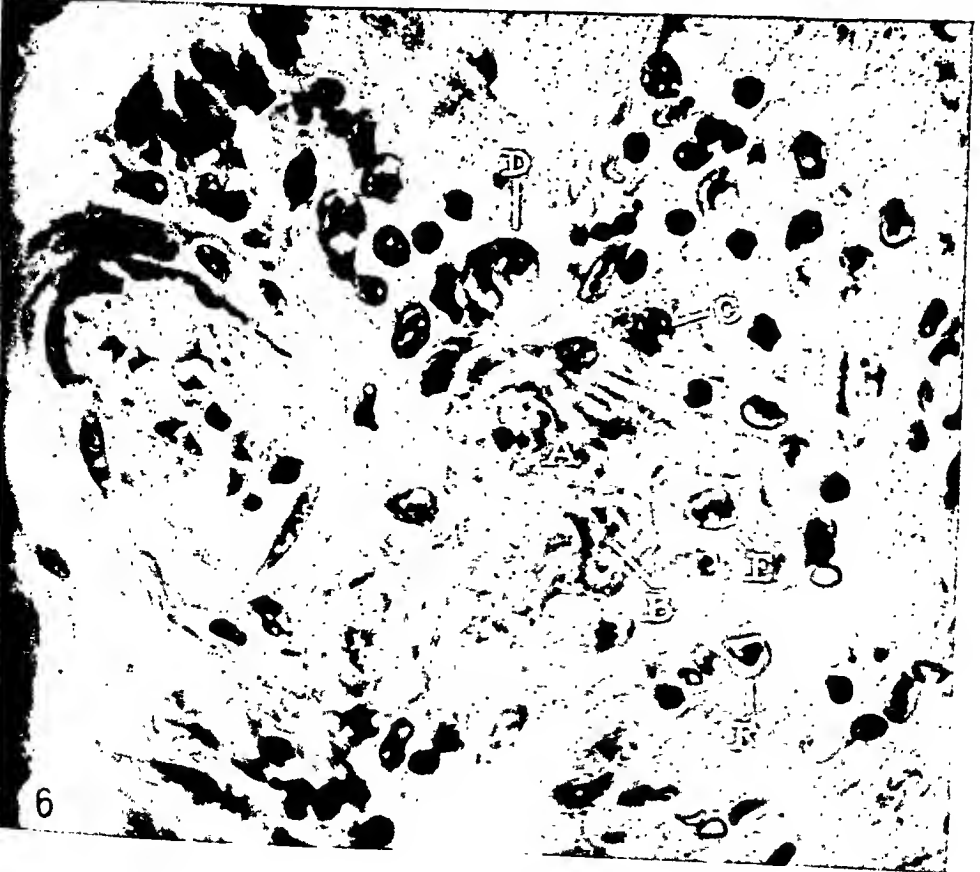
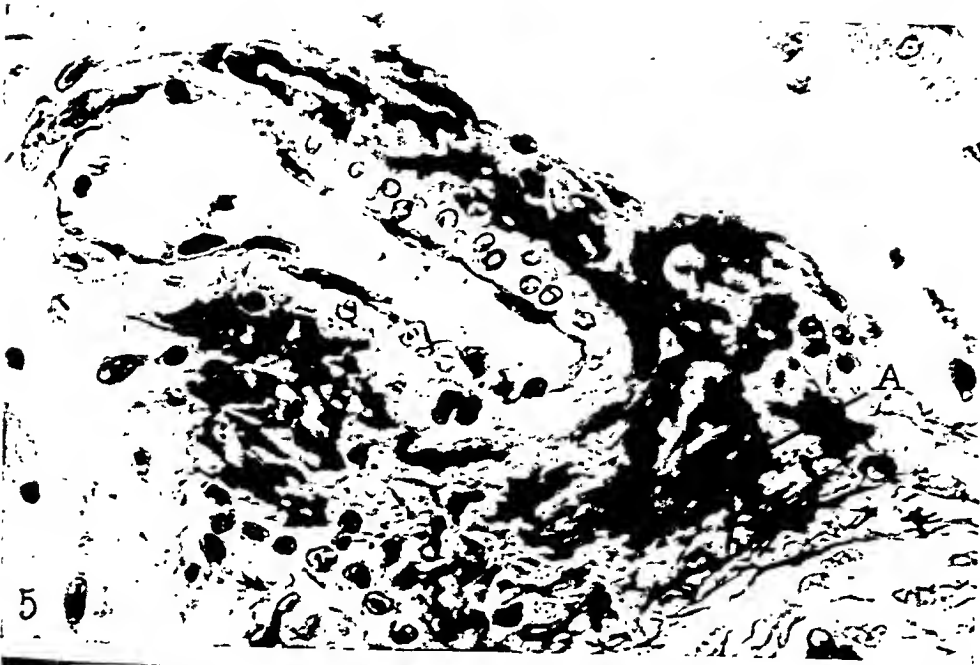


PLATE 39

FIG. 5. Rabbit 71-77,—artery in left ventricle. *A*, frankly fibrinoid collagen, bordered by granuloma cells, in adventitia and paraadventitia; panarteritis nodosa absent. Weigert-hematoxylin and eosin. $\times 743$.

FIG. 6. Rabbit 71-77,—adventitial and paraadventitial coronal granuloma in inter-ventricular septum. *A*, center of focus of frankly fibrinoid collagen; *B* and *C*, indistinct cell masses; *D*, cell with 3 nuclei; *E*, cell with fibrocytoid nucleus; *F*, cell with owl-eyed nucleus; many cells have indistinct cytoplasmic outlines; panarteritis nodosa absent. Hematoxylin and eosin. $\times 861$.

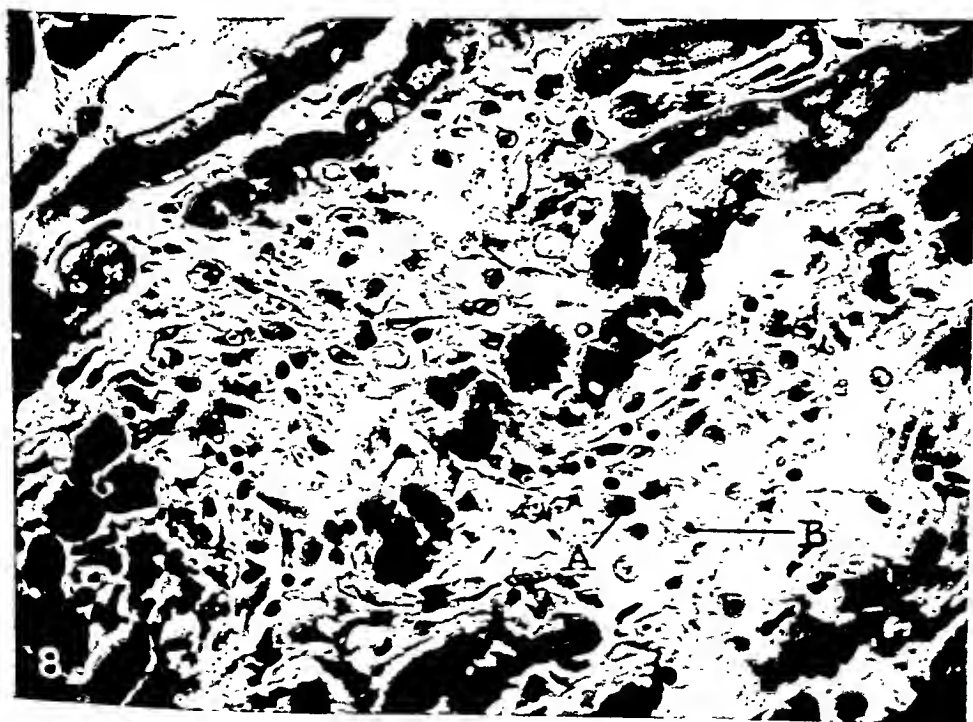
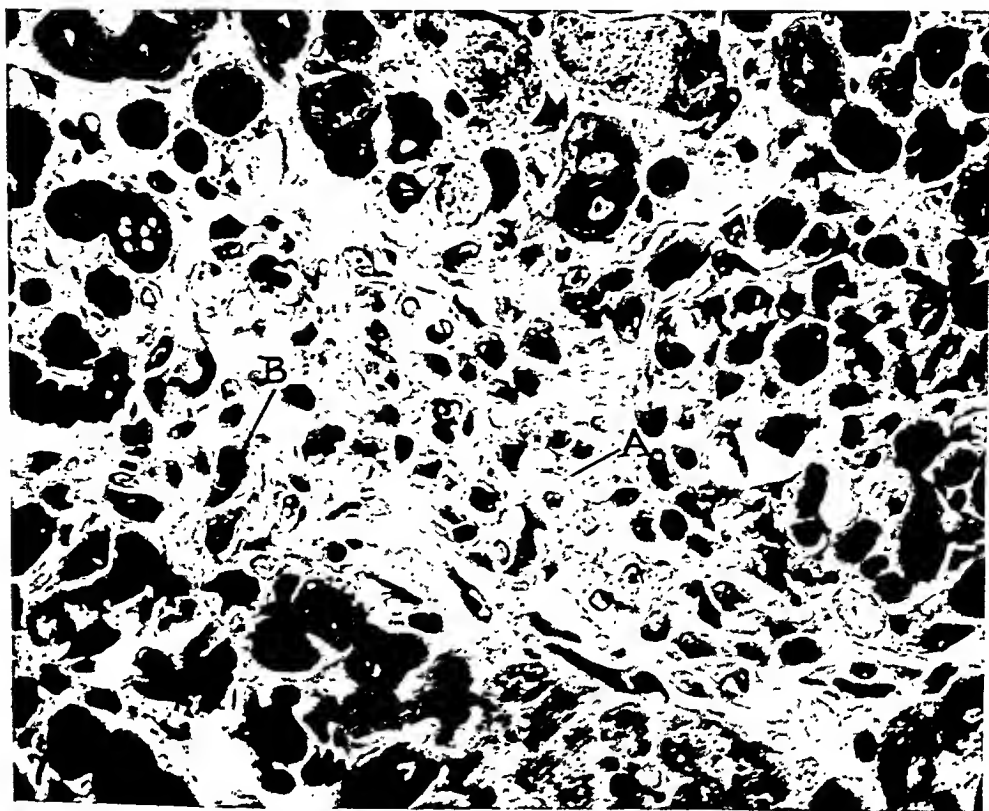


(Murphy and Swift: Induction of cardiac lesions)

PLATE 40

FIG. 7. Rabbit 71-80 (see Table III),—sacrificed 10 days after last of 6 infections; autopsy blood cultures negative; reticular myocardial granuloma in interventricular septum. Cells interspersed in interlacing network of (A) swollen collagen fibers; B, cell with abundant basophilic cytoplasm; vacuolation of nuclei and cytoplasm of adjacent myofibers. Giemsa stain. $\times 621$.

FIG. 8. Rabbit 71-80,—two mosaic myocardial granulomata in left ventricle; granuloma cells lodged between collagen masses. A, cell with 3 nuclei; B, cell with pyknotic nucleus and abundant raggedly outlined cytoplasm; most cells have indistinct cytoplasmic outlines; disintegration of adjacent myofibers. Hematoxylin and eosin. $\times 404$.

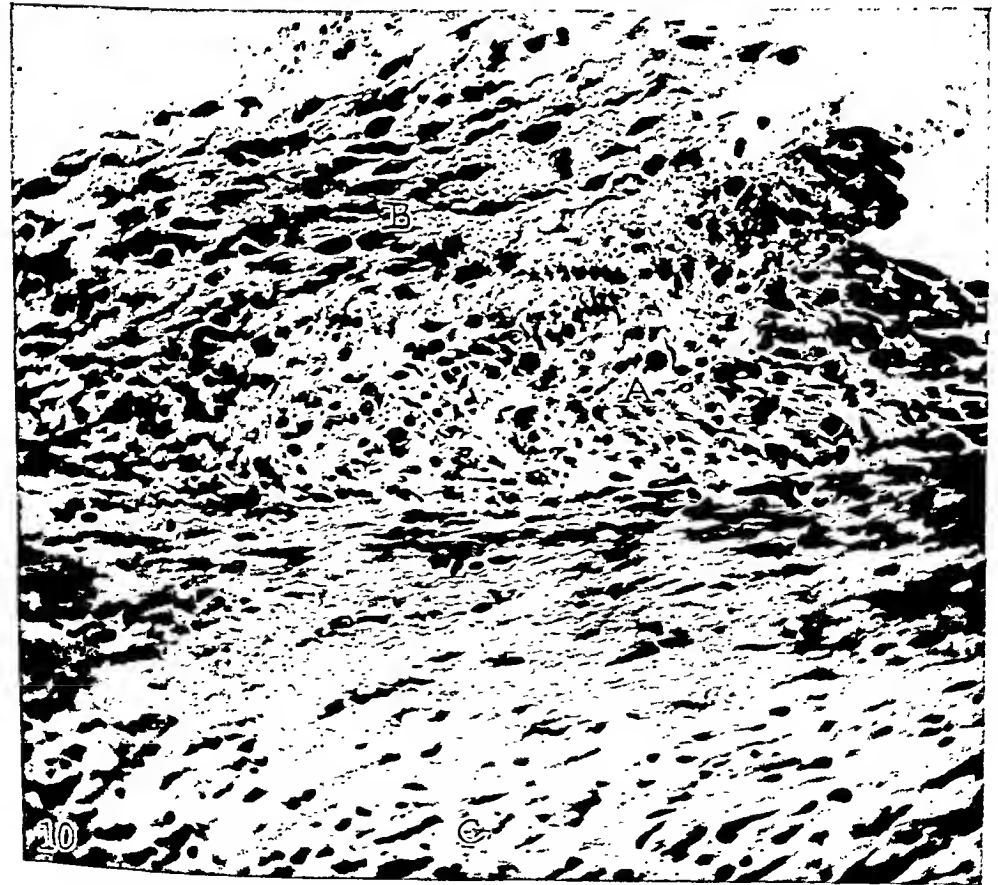
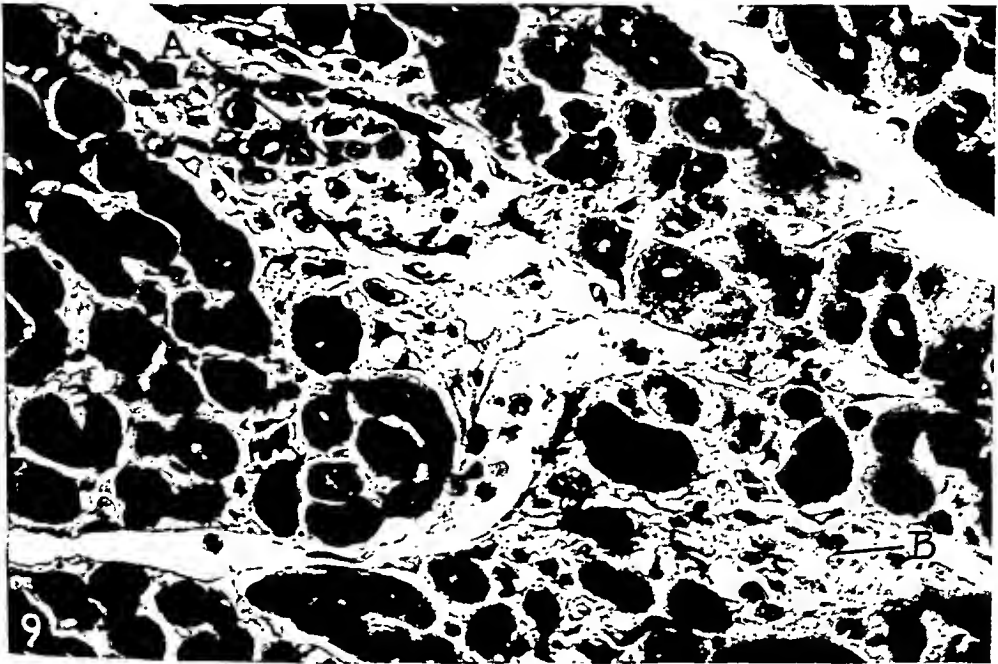


(Murphy and Swift Induction of cardiac lesions)

PLATE 41

FIG. 9. Rabbit 71-80 (see Table III),—left ventricle; *A* and *B*, 2 mosaic myocardial granulomata; foci of frankly fibrinoid collagen in granuloma *A*. Hematoxylin and eosin. $\times 465$.

FIG. 10. Rabbit 71-77,—endocardial nodule on chorda tendineae at mitral leaflet attachment. Numerous multinucleated cells surrounding *A*; many cells with basophilic cytoplasmic streamers surrounding *B*; interstitial inflammation, *C*. Giemsa. $\times 255$.

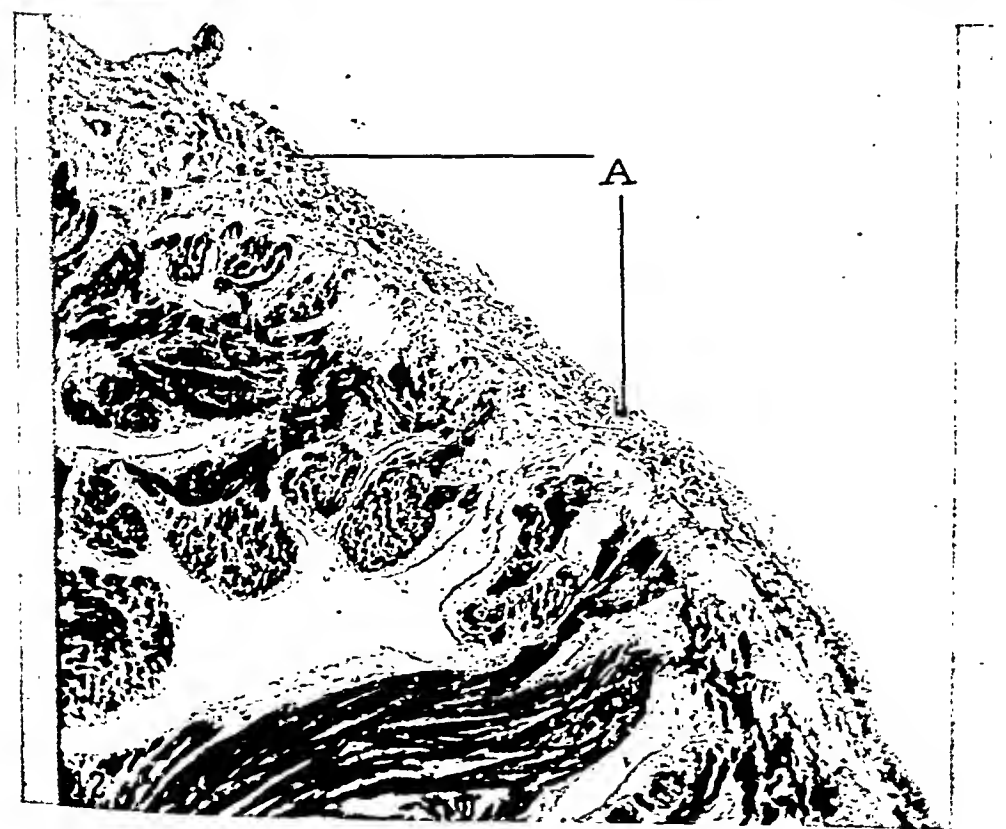
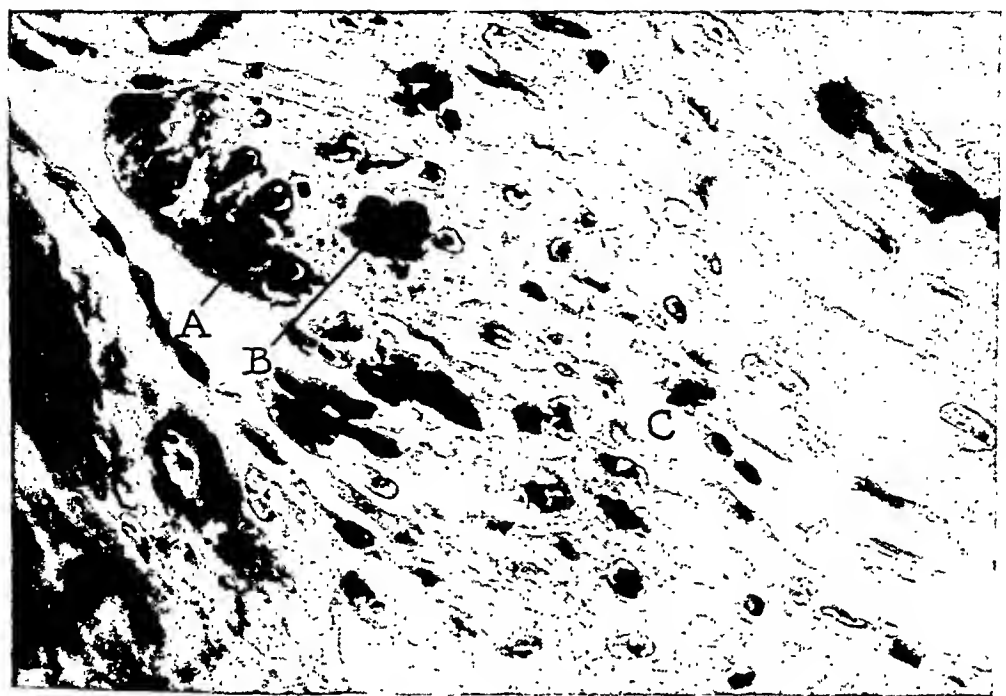


(Murphy and Swift: Induction of cardiac lesions)

PLATE 42

FIG. 11. Rabbit 70-58 (see Table I),—died 13 days after last of 8 infections; negative blood cultures 2 days prior to and at autopsy. Left ventricle; mosaic nodular granuloma arising from thin walled vein; granuloma cells lodged between frankly fibrinoid masses (*A*); cell at *B* has 2 nuclei; several cells with owl-eyed nuclei; axially arranged cells surrounding *C* have streamers of cytoplasm; dissolution of adjacent myofibers. Masson trichrome stain. $\times 659$.

FIG. 12. Rabbit 70-71,—sacrificed 16 days after last of 8 infections. Left auricle; *A*, epi- and subepicardial collagen converted into frankly fibrinoid material; Mallory aniline blue stain. $\times 127$.



(Murphy and Swift: Induction of cardiac lesions)

THE STRUCTURE OF ELASTIC TISSUE AS STUDIED WITH THE ELECTRON MICROSCOPE*

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PLATES 43 TO 47

(Received for publication, March 14, 1949)

In contrast to its near relative, collagen, elastic tissue has thus far received meager attention with respect to fine structure analysis. Both morphologically and chemically this tissue has in the past been generally considered to be a relatively homogeneous entity. The present study indicates this to be highly unlikely. At least two distinguishable entities have been observed.

Previous investigations revealed no distinctive patterns of structure either by x-ray diffraction (1, 8) or electron microscopy (17). Collagen, on the other hand, yielded x-ray patterns indicating a high degree of organization (1, 2, 9). A complex axial repeating period with considerable detail of intraperiod structure was demonstrated in the fibrils with the electron microscope (5, 13, 15).

Astbury (1) observed a typical collagen x-ray diffraction pattern in stretched ligamentum nuchae, which, however, was reduced to amorphous rings after autoclaving (to remove the large amount of collagen known to be present). W. J. Schmidt (12) described an increase in double refraction on stretching the ligamentum presumably due to orientation of fibrous units.

Wolpers (17) found it necessary to treat elastic tissue with pepsin in acid for 24 hours before he could obtain fibers of a size suitable for study with the electron microscope. This partially digested elastin¹ from ligamentum nuchae revealed large, branching, amorphous fibers varying in width from 2,500 Å to 200 Å, the very smallest seen being 80 Å. No axial periodicity was noted. Fixation in osmic acid resulted in a fine longitudinal fibrillation which Wolpers ascribed to the action of the fixative and not intrinsic to the actual structure. Orcein, a stain considered relatively specific for elastic fibers, was observed to deposit on these fibers in the form of small flakes. The elastin of the mouse aorta, after acid pepsin digestion, osmic acid fixation, and sonic fragmentation, appeared as thin, fenestrated laminae with numerous, short, stubby fibers protruding from the surface.

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¹ The term "elastin" will be used synonymously with elastic tissue in this report and will not define a particular protein.

Meyer and Ferri (11) from thermoelastic considerations, deduced that elastin is probably made up of highly contorted polymeric chains bonded laterally by only few links. Stretching the fiber orients these units in an axial direction thereby reducing the entropy and increasing the internal energy. Thermal motion produces the elastic recoil. Jordan Lloyd (7) agreed with this interpretation and added that the structure differs from rubber in that the peptide linkages of the elastin polymeric chains have polar properties which, by cross-linking, serve to maintain the extended state in stretched and dried elastic fibers. Imbibition of water is necessary to spread the chains apart, thus permitting thermal contraction whereas rubber, being entirely apolar, will not "lock" in the extended state when dry at ordinary temperature. McCartney (10) observed that ligamentum nuchae when freed of its collagen by peptic digestion contracts linearly with increase in temperature and returns along the same straight line to its original length on cooling. This suggests no extensive alteration in structure when the tissue is heated to 100°C. in water.

Chemical analysis of ligamentum nuchae by Stein and Miller (16) indicated roughly one in thirty amino acid residues to be polar (excluding glycine) in contrast to about 50 per cent for collagen.

Methods

The swim bladder of the carp, the aorta of the adult rabbit, and 2 day old rat, that from a human being 5 years old, and ligamentum nuchae of the cow were used as sources of elastin in this study. The swim bladder² proved to be a particularly suitable source of material because the collagen (ichthyocol) was readily removed by heating to 60°C. in dilute acetic acid, although nearly all of it could be removed by the dilute acid without heat. Mild fragmentation procedures reduced the elastin fibers to suitable size. Elastic tissue of the ligament and aorta posed difficult problems of fragmentation.

Metal shadowing was found to be the most suitable preparative adjuvant although heavy metal staining (6) was also attempted. The same preparative procedures were used for all tissues except for modifications required by certain special characteristics of each material.

The elastin of the carp swim bladder was prepared in the following manner. The tunic was stripped from the fresh bladder, washed well with water, cut into small pieces, and allowed to stand overnight in 1 per cent acetic acid in the refrigerator. The collagen swells greatly forming a viscous solution which is readily filtered off through silk bolting cloth. The gelatinous residue was then placed in dilute acetic acid and fragmented for 10 seconds in a Waring blender. The suspension, which is fairly viscous, was centrifuged to sediment the finely dispersed elastin. Repeated washing in the centrifuge in dilute acid removed practically all the "dissolved" collagen, leaving a residue which formed a fine, cloudy suspension on agitation. However, to be certain that no collagen filaments (14) were present to confuse the picture, the suspension was boiled for 30 minutes. Swim bladder collagen is readily gelatinized at this temperature. Heating greatly reduces the viscosity of the suspension and facilitates sedimentation of the elastin fibers. As will be described later, heat and dilute acid do not seem to affect the observed structure of intact elastin. The finely comminuted elastin was then washed several times in the centrifuge and resuspended in distilled water.

The aortic tissues and bovine ligamentum nuchae were prepared in a somewhat different fashion. The fresh, unfixed tissues were sectioned with the freezing microtome. One portion was boiled for 1 hour in 1 per cent acetic acid to remove the collagen, while a second portion was not heated so as to permit study of the relationship between collagen and elastin.

² Faure-Fremiet and Garrault (4) give an excellent histological description of the fish swim bladder.

In order to reduce the elastic fibers to a size suitable for electron microscopy preparations were further fragmented in a sonic (9 kc. magnetostriction type) oscillator.

The resulting suspensions were prepared for examination in the usual manner by depositing drops on supporting films of conventional nickel specimen grids and blotting them off with filter paper after about 2 minutes. Some preparations were examined without further treatment. Most were shadowed with 8 mg. of chromium or 10 mg. of uranium at an angle of 10° and a filament to specimen distance of 20 cm.

The effect of digestion with crystalline trypsin (Armour) was studied by incubating portions of each material, boiled and not heated, in 0.1 per cent enzyme buffered to pH 8-8.4 with NaHCO_3 at 37°C . for periods ranging from $2\frac{1}{2}$ to 24 hours under toluene. These suspensions were then washed in the refrigerated centrifuge at 4,000 R.P.M. for 1 hour with distilled water to remove the dissolved solids. Specimens were prepared for electron microscopy as described, except that the drops were allowed to remain on the grids for at least 10 minutes before blotting. Buffered controls without enzyme were also examined.

The influence of temperature on the fibrous elements released by tryptic digestion was investigated by heating portions of the washed, trypsinized suspensions in a water bath at temperature intervals of about 10°C . ranging from 40° to 100° for 30 minutes.

Pure crystalline trypsin solutions were examined to rule out possible artifacts from this source.

The effect of pH on the washed, trypsinized suspensions was studied in the range 2.5 to 10.5 at nearly unit intervals and room temperature. Unbuffered acetic acid and ammonium hydroxide were used to make up the pH series. This experiment was not performed on the rat aorta.

To observe the effect of formalin fixation portions of boiled ligamentum nuchae elastic tissues were fixed in 10 per cent neutral formalin for 7 days, incubated in trypsin, and prepared for electron microscopy as described.

An RCA type EMU electron microscope was used in this study.

RESULTS

Tissue of the Fish Swim Bladder.—The elastic tissue of the swim bladder tunic is extremely friable, requiring only 10 seconds in the Waring blender to reduce it to a fine, cloudy suspension. Electron microscopy revealed the suspended particles to be long, contorted, branching fibers ranging from about 300 Å to 5,000 Å in width and often measuring many microns in length. In addition to the fibers there were numerous clumps of coarse, granular material. Excessive blending destroyed most of the fibers leaving only granular masses. In some preparations which had not been heated but only washed in dilute acetic acid, extremely fine filaments were present in the background which disappeared on heating to 40°C .; these were most likely ichthyocol (14). No intact collagen fibrils were found in boiled preparations. The following descriptions are based on electron micrographs of chromium-shadowed specimens.

The fibers appeared to be irregularly flattened on the supporting film (possibly a drying effect) and usually showed a roughly parallel and often coarsely interlaced fibrillation. These fibrillar units ranged in width from about 300 Å to 1,000 Å and appeared to be embedded in an amorphous matrix. The latter was observed to flatten out at the edges and often assumed a finely packed texture (Fig. 1). The "grain" of this texture, which was not always observ-

able, ran both parallel and perpendicular to the long axis in different fibers. Branching of a fiber was characterized by a separation of the fibrils (see Text-fig. 1) with a stretching and thinning of the binding matrix at the bifurcation. Occasionally a frayed fiber would show the presence of fibrous units no more than 100 A. in width. There was no evidence of a true axial periodicity.

Boiling for 30 minutes in 1 per cent acetic acid had no effect on the observable structure.

Observations of unshadowed fibers added nothing to the pattern and the limited number of experiments with osmic and phosphotungstic acid staining were unproductive.

Aorta of the Adult Rabbit, Rat, and Human Being.—Boiling frozen sections of aorta in 1 per cent acetic acid destroys the collagen and most probably all other structural elements except the elastin. Fragmentation by freeze-sectioning and high speed homogenization produced very few tissue fragments small enough for electron microscopy. However, strong sonic vibrations disrupted the strongly coherent elastic membranes, producing fibers of size suitable for study (Fig. 4). These closely resembled the elastic fibers found in the tissue of the swim bladder. Whether the fibers observed were part of the lamina or actually represented the interlamellar fibers could not be determined. Considerable amounts of granular material were also present. No fine filaments were found. Large numbers of typically striated collagen fibrils were associated with the elastic fibers in the unheated preparations and often appeared to protrude from the fragments of elastin.

Ligamentum Nuchae of the Cow.—The long, thick fibers of this tissue were extremely resistant to fragmentation, more so than the fibers of the aortic elastica. Prolonged treatment with 9 kc. sonic waves was required to produce even a few suitable fibers. The fragments were usually small irregular chunks rather than fibrous units. Apparently these fibers are as strongly coherent in the lateral direction as they are longitudinally. The few fibers obtained were too thick and dense to reveal any detailed structure. In one rare case of a much flattened, frayed elastic fiber, numerous fine filaments about 100 A in width were found lying parallel with the fiber axis.

The Effect of Trypsin.—The most striking result of tryptic digestion was the appearance of many fine threads of constant cross-section in all fields examined and in preparations of all three tissue types. They were barely visible in unshadowed preparations. Threads released from the three different types of elastic tissue were indistinguishable with regard to their morphology (Figs. 6, 7, and 9).

In particularly clean preparations two morphological forms of threads were observed. One type found in varying numbers in different preparations of the same tissues was a tightly and evenly coiled double helix formed by the twining of two thin, apparently smooth filaments. In the same fields many uncoiled

filaments could be found usually lying in closely associated, parallel pairs and usually much longer than the coiled forms (Figs. 8 to 10). There were many instances observed in which a coiled thread was transformed abruptly into a parallel pair of smooth filaments, and in some cases, as shown in Fig. 7, only a single filament would project beyond the coil. Occasionally both ends of a coiled thread would continue as a pair of parallel filaments. The widths (actually height above the supporting film) of 350 filaments and threads obtained from ligamentum nuchae were determined from the shadow length, the only selection factor being the perpendicularity of the long axis of the thread to the direction of the shadow. A characteristic width of about 120 Å was calculated for the coiled threads and approximately 70 Å for the individual filaments. These figures apply as well to the threads and filaments obtained from the other tissues studied. Direct measurements of widths on an unshadowed preparation (which is not entirely satisfactory because of low contrast) roughly confirmed these figures.

The pitch of the helix in 120 measured threads from ligamentum nuchae ranged from 470 Å to 580 Å with a peak at about 530 Å. Again, these figures were approximated by those for the aortic threads. These fibrous elements were usually straight or gently curved but never acutely kinked. No discrete axial periodicity was resolved other than that produced by the observed coiling.

Threads and filaments were never found in preparations not digested by trypsin.

Effects of Temperature, Formalin Fixation, and pH.—Heating threads and filaments of each of the three forms of elastic tissue suspended in distilled water resulted in their disappearance in the temperature range 70–85°C. Boiling the tissue in dilute acid prior to tryptic digestion did not alter the critical temperature.

Formalin-fixed ligamentum nuchae which had been boiled in dilute acid prior to fixation was digested in trypsin in a manner identical with that described for the fresh material. Typical threads and filaments were found. No further characterization was attempted at this time.

The influence of pH on the state of aggregation and morphology of the threads obtained from ligamentum nuchae was studied. At pH 3.0 only amorphous clumps of material often resembling small, flat discs were observed. At about pH 3.6 these clumps were larger, flatter, more irregular, and more granular (Fig. 13). At pH 5.1 the aggregates appeared coarsely reticular with short, stubby, nodular threads protruding from the edges. No obvious coiling was observed but there seemed to be a fine, granular debris covering everything which obscured detail (Fig. 12). No free filaments were observed. At increasingly higher pH the threads were increasingly longer and finer. At pH 5.7 some free threads were observed, plus many loose aggregates. At pH 6.2 very little aggregation was observed and characteristic threads and filaments

were present. Coiled forms were found in large numbers in the pH range 6.1 to 9.1 (Figs. 7 and 8). If a suspension of threads were allowed to stand for about 24 hours at neutral pH, clumping was also observed.

The ratio of coiled threads to smooth filaments diminished with increasing alkalinity; at pH 9.8 no coiled threads could be found (Fig. 11). Increase in pH had no observable influence on the pitch of the helices; they were either coiled with the characteristic pitch or completely uncoiled. Boiling the tissue in dilute acid prior to digestion did not alter the pH effects. Less detailed experiments performed on the threads of rabbit aorta and fish swim bladder indicated a similar behavior pattern.

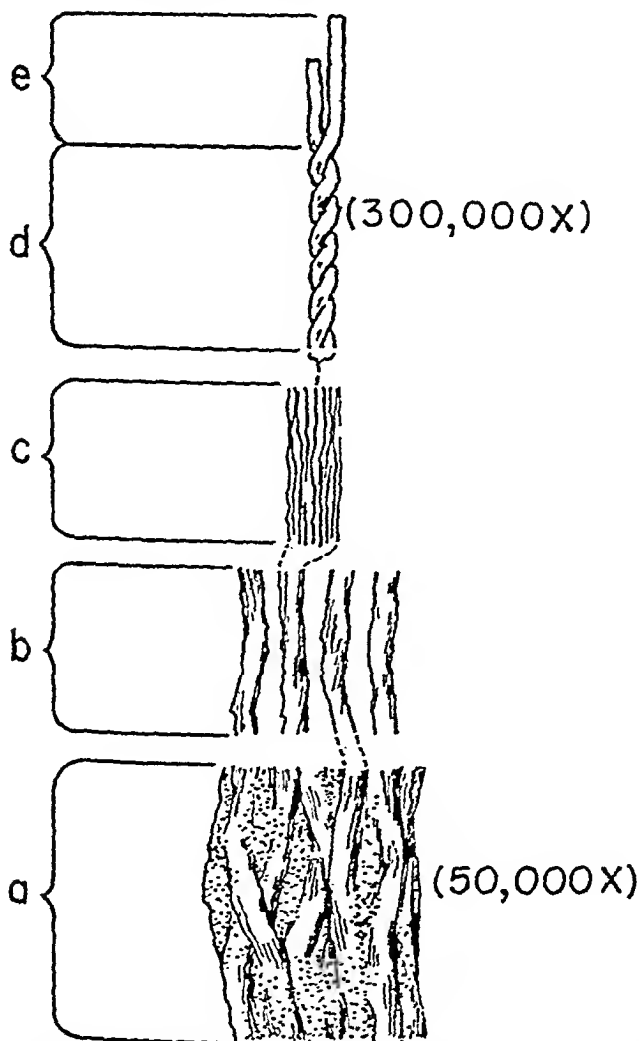
Partially Digested Fibers.—Trypsinized elastin of the swim bladder tunic and aorta revealed numerous fibers of a size well suited for electron microscopy. Many of the partially digested swim bladder fibers appeared to be flattened amorphous, and moth-eaten (Fig. 3). In many cases, however, the amorphous matrix seemed to have been partially stripped away, revealing nearly naked fibrils (Figs. 2 and 5). In rare instances partial fragmentation of these fibrils revealed thinner units of about the size of the free threads. However, even here there seemed to be enough amorphous material present to obscure any details such as coiling. The characteristic coiled threads have not yet been observed in either intact or partially digested fibers.

DISCUSSION

From the data at hand one may construct a tentative model of the architecture of the elastic fiber (see Text-fig. 1). At least two distinct chemical and morphological components, namely the threads and the amorphous binding substance, are associated to form a fiber which is capable of long range elasticity, great mechanical strength, and refractiveness to boiling water, dilute acids or alkalis. Collagen is present as an incidental component, probably incorporated in the fiber extracellularly during its formation as suggested in Bloom's study of elastic fibers in tissue culture (3). The threads are arranged in long, roughly parallel bundles, such as observed in partially digested fibers, which may or may not course the full length of the fiber. These bundles are probably both infiltrated with and embedded in the trypsin-sensitive, amorphous binding matrix. The relative proportions of matrix and threads cannot readily be determined by electron microscopy.

Matrix.—Whether this substance is amorphous in a rigorous sense or is actually a tangled mass of long macromolecular chains like rubber, remains to be determined. The matrix substance is apparently responsible for the heat resistance of the intact fiber since the naked threads (released by trypsin) are relatively heat-labile. The matrix is probably bound to the filaments in such a way as to prevent their destruction by heat. Probably the great difference in friability between the intact fibers of the different elastic tissues is determined by the tensile strength of the matrix.

It is interesting to note that formalin fixation apparently does not prevent the digestion of the matrix material by trypsin. This fact may be of significance in considering the chemical nature of the matrix.



TEXT-FIG. 1. Schematic representation of the structure of the elastic fiber (collagen fibrils omitted). (a) Undigested fiber showing fibrils imbedded in amorphous matrix (see Fig. 4). (b) Partially digested fiber showing fibrils stripped of matrix (see Figs. 2 and 5). (c) Single fibril, enlarged, revealing it to be a bundle of threads. (d) Single coiled thread, greatly enlarged (see Figs. 8 and 9). (e) Individual filaments which, when twined, form the thread (see Figs. 10 to 11).

The Fibrous Component.—The threads obtained from the different elastic tissues are similar in many respects; e.g., morphology, heat sensitivity, and

reaction to pH. This suggests that these characteristic units are a uniform constituent of elastic tissue.

The tendency to aggregate on the acid side and to fray into finer threads at higher pH suggests an isoelectric point in the acid range. Stein and Miller (16) from electrophoretic studies of purified, finely comminuted, whole elastic tissue estimated an isoelectric point of 4.8. At the present time there are no further chemical data to characterize these structures more fully.

The coiling of these fine fibrous elements is of great interest because of its possible rôle in elastic and contractile processes. Indeed, the structural pattern described here has all the components of the molecular model for elastin postulated from thermodynamic considerations by Meyer and Ferri (11); *i.e.*, a compressed, coiled spring held under tension by a stretched elastic band. If this analogy were pursued, one would expect that the observed coiled threads are compressed in the native state and that tryptic digestion frees them from the restraining force of the matrix, thus permitting a large increase in pitch. It is therefore important to demonstrate conclusively whether the coiled structure is characteristic of the intact tissue and not a result of manipulation. The chance that the coiled threads are artifacts produced by the axial rotation of adjacent filaments while in suspension is remote. The coils are always tight and regular, and show little variation in pitch even in different forms of elastic tissue; moreover intermediary stages of coiling have never been observed. Another bit of evidence is the appearance of large numbers of filaments and the disappearance of coiled threads with a rise in pH and the inability to reunite these threads into coils on lowering the pH. Only haphazard lateral aggregation with some irregular twining occurs. The assumption here is that the smooth, thin filaments are produced by uncoiling of the threads and are not a separate species.

The large numbers of parallel pairs of filaments which sometimes abruptly twine to form a short segment of typically coiled thread suggest that two filaments form a single thread. Because the individual uncoiled filaments are nearly always very much longer than the coiled threads—longer than one would expect to result from simple uncoiling of the thread—there exists the possibility of a much finer, molecular coiling in the filament which is also smoothed out when the thread uncoils. It is evident, however, that considerably more data are needed to answer conclusively the basic questions as to the morphology of the threads in the native state and the respective rôles played by the two components in elastic behavior.

Further experimentation with the electron microscope and physical chemical techniques on the liberated threads, and also x-ray diffraction and polarized light studies on the intact tissue, are needed to provide the essential evidence. With regard to early polarized light studies (12) the correlation between the observed isotropy of relaxed elastic tissue and the structures described here is obscure at present.

Stein and Miller (16) have made an amino acid analysis of "purified" elastin. However, they have treated this substance as a single homogeneous protein even to the extent of working out the amino acid "frequencies" according to the Bergmann-Niemann "periodicity theory." The results described here demonstrate the presence of at least two components. It is therefore highly desirable that new amino acid analyses be made on the individual components. This should now be feasible since the present work indicates procedures by which the two fractions may be separated.

The author wishes to thank Mrs. Mary Frances Simmons and Mr. James Wilson for their valuable technical assistance.

CONCLUSIONS

Electron microscope examination of fragmented elastic tissue obtained from fish swim bladder, bovine ligamentum nuchae, and aortas of various mammals, including man, reveals characteristically formed fibers and much amorphous material. Boiling in dilute acid destroys the associated collagen but does not obviously alter the elastic tissue.

Digestion in crystalline trypsin of either boiled or unheated tissue from any of the above-mentioned sources causes the release of thin threads ranging in length from $0.1\ \mu$ to many microns. A large proportion of these threads are evenly and tightly coiled double helices formed from at least two interlacing filaments and measuring about 120 Å in width. The distance between coils ranges from about 470 to 590 Å. The individual smooth filaments, many of which are present in parallel pairs, measure approximately 70 Å in width.

Raising the pH of a neutral suspension of threads from ligamentum nuchae lowers the ratio of helical threads to uncoiled filaments, whereas lowering the pH with acetic acid results in clumping of threads with complete loss of identity at about pH 3.6.

Threads and filaments obtained from all sources studied were destroyed in the temperature range 75–85°C. at pH 7.

It is concluded that the elastic fiber is a two component system composed of bundles of trypsin-resistant threads of characteristic form and size plus a trypsin-sensitive, heat-resistant "amorphous" binding matrix.

The possible relationship of this structure to the elastic properties of the tissue is discussed.

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EXPLANATION OF PLATES

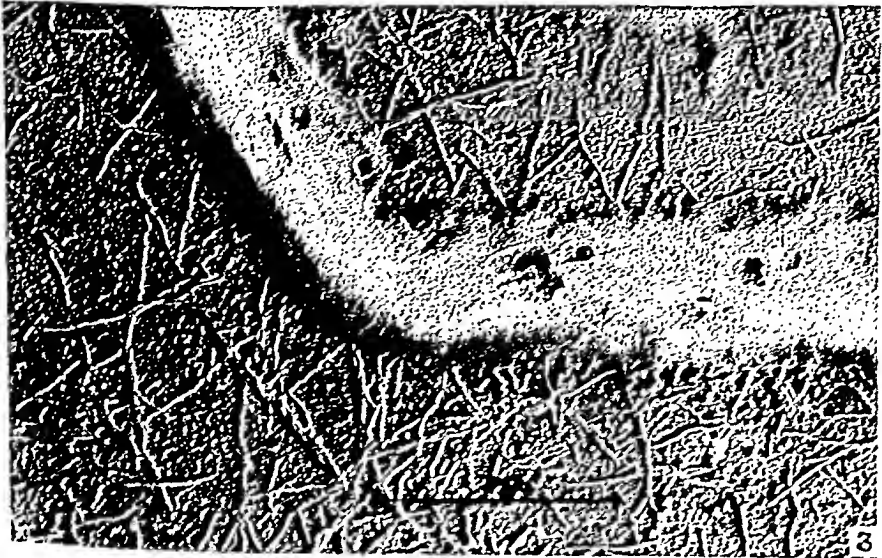
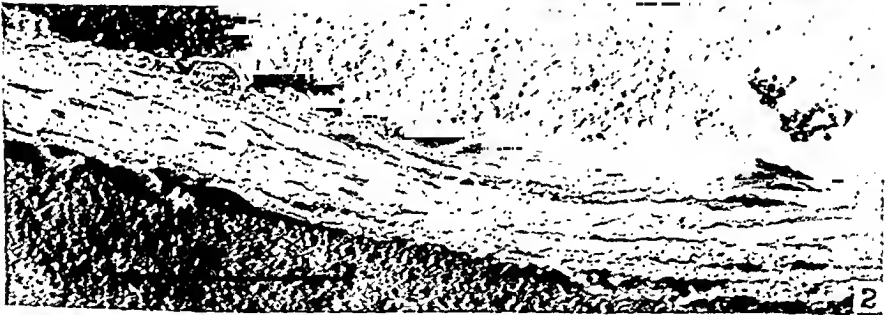
PLATE 43

Fish swim bladder elastin heated at 60°C. in dilute acid. Fragmented and washed. All specimens were chromium-shadowed.

FIG. 1. Fibers deposited on grid from aqueous suspension. $\times 14,000$.

FIG. 2. Fiber which has been partially digested with crystalline trypsin. Much of the amorphous matrix has been removed revealing the fibrils. $\times 32,000$.

FIG. 3. After digestion with trypsin filaments released by the enzyme can be seen. An amorphous fragment of a partially digested fiber remains. $\times 31,000$.



(Gross: Structure of elastic tissue)

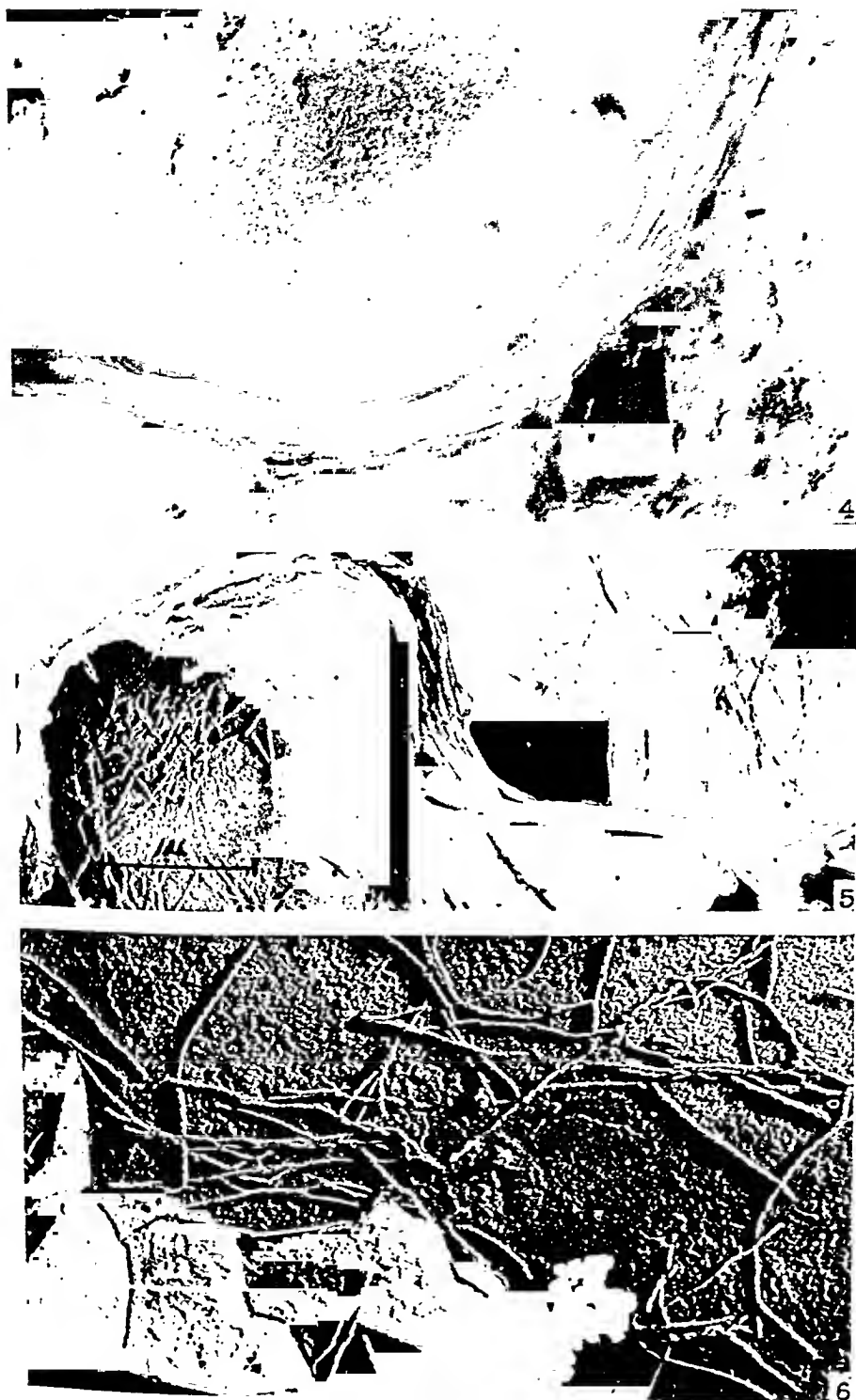
PLATE 44

Adult rabbit aorta boiled in 1.0 per cent acetic acid, freeze-sectioned. All preparations were chromium-shadowed. $\times 22,000$.

FIG. 4. Fiber from suspension produced by treating tissue sections with sonic oscillations. Coarse fibrillation of the structure is noted, plus considerable amounts of amorphous material. $\times 22,000$.

FIG. 5. Partial tryptic digestion of an aortic elastin fiber, revealing the roughly parallel fibrils which are believed to be bundles of threads. Many free threads can be seen in the background. $\times 21,000$.

FIG. 6. Aortic elastin threads released by trypsin. Characteristically coiled threads can be seen in addition to some uncoiled filaments. The large, dense lobular mass is an unidentifiable contamination. $\times 28,000$.



(Gross: Structure of elastic tissue)

PLATE 45

FIG. 7. Threads and filaments released from bovine ligamentum nuchae by tryptic digestion. Numerous coiled threads are observed, along with thinner uncoiled filaments which in some cases can be seen to project individually beyond the coiled regions. Shadowed with chromium. $\times 23,000$.

FIG. 8. Higher magnification of coiled threads and filaments. Shadowed with chromium. $\times 42,000$.



(Gross: Structure of elastic tissue)

PLATE 46

FIG. 9. Threads and filaments released by trypsin from the elastin of the fish swim bladder tunic. A single coiled thread is seen in this field. The filaments are much longer than the threads and in most cases are paired. Shadowed with chromium. $\times 29,200$.

FIG. 10. Human aorta from 5 year old girl. Not preheated. Digested with trypsin. pH raised to 10.0. Thin, uncoiled filaments are observed. Coiled threads have not thus far been found at this pH. Two typical intact collagen fibrils are also present. Shadowed with uranium. Pebbly background represents unusually coarse collodion film structure. $\times 36,100$.

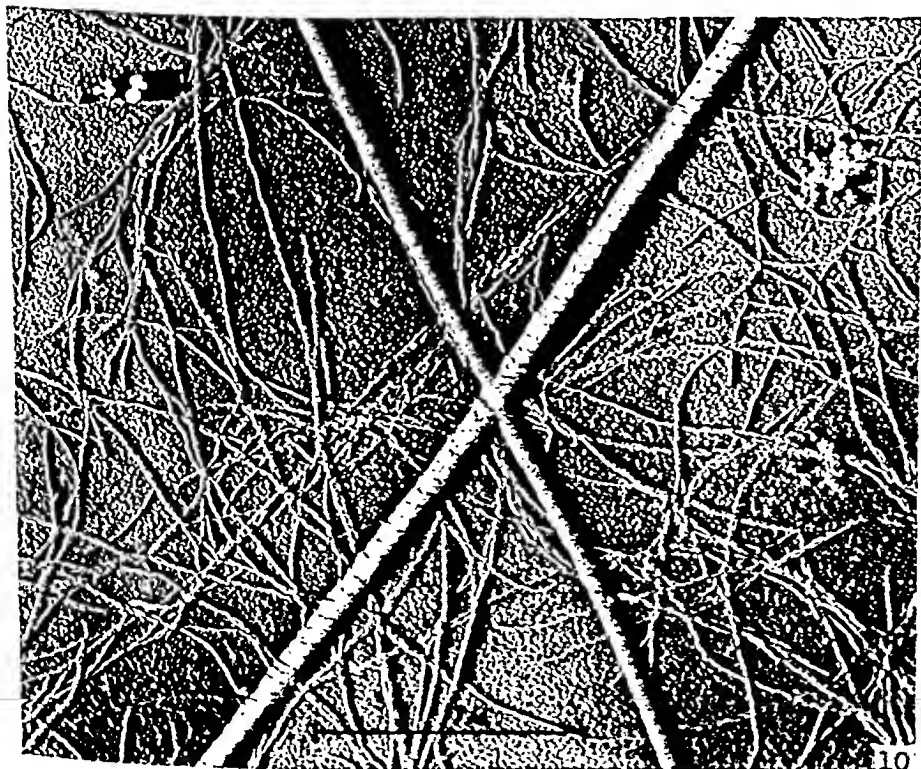


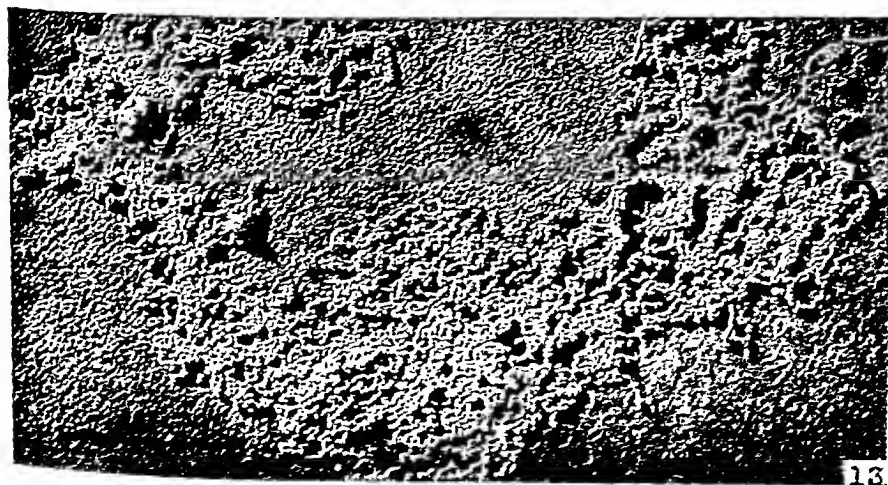
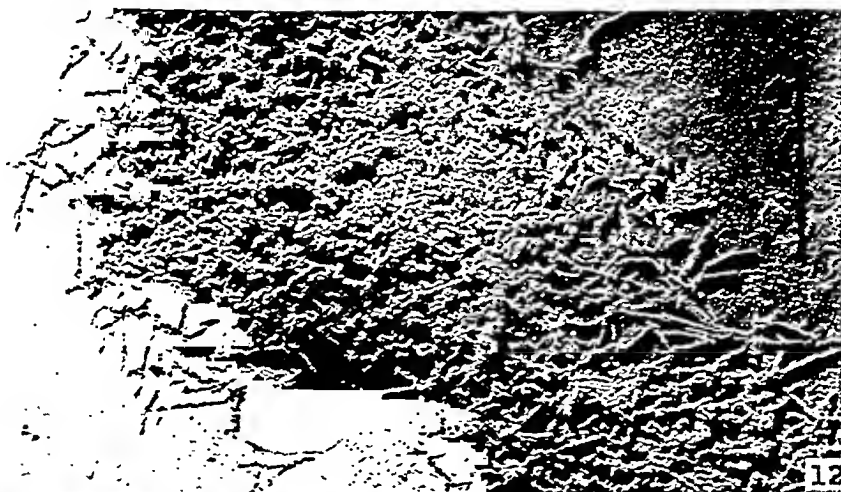
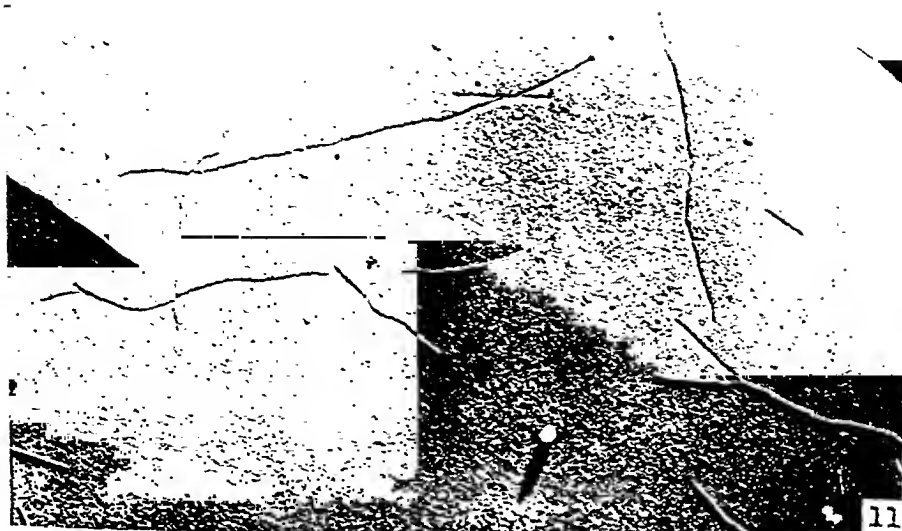
PLATE 47

Influence of pH on ligamentum nuchae threads. All specimens were chromium-shadowed.

FIG. 11. pH 9.8. Only individual and occasionally paired filaments are observed. No coiled threads were seen. $\times 22,500$.

FIG. 12. pH 5.1. Clump of short, irregularly nodular threads. Coiling was not resolved. $\times 24,750$.

FIG. 13. pH 3.6. Granular mass in which individual threads are no longer identifiable. $\times 21,750$.



(Gross: Structure of elastic tissue)

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only two out of twenty one of the primary tumors formed secondary growths—a small nodule in the liver in one case, two small nodules in the liver in the other. The results in the group maintained at 28°C. are strikingly different. Here six out of eighteen tumors became disseminated, and the secondary growths were extensive. A representative example of such metastasis is shown in Figs. 9 to 11. The liver and lungs of the frog were so riddled with secondary tumors that the organs were almost completely replaced. It is noteworthy that in this as well as in other animals of the group the secondary tumors were of approximately uniform size, suggesting that they had become established at about the same time.

In twenty six control frogs maintained under fairly natural conditions at 18°C. for an average of 140 days only one secondary tumor in the liver was encountered.

TABLE II
Effect of Temperature on Incidence of Metastasis: Summary of Experiments

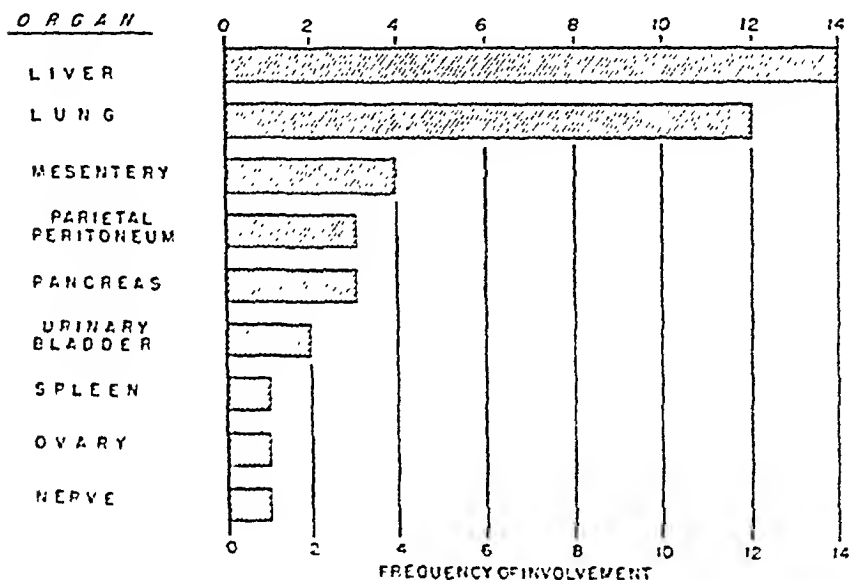
Temperature	No. of animals	Average duration of exposure	No. with metastatic tumors	Per cent with metastatic tumors
		days		
7°	21	98	2	6
18°	26 } 47	130	1 } 3	
28° (1st series)	18	47	6	54
28° (2nd series)	17 } 35	50	13 } 19	

Second Series of Experiments.—An even more pronounced effect of prolonged exposure to elevated temperature was found when well nourished frogs were used (group D). Their exposure averaged 50 days; *i.e.* approximately the same as in the first series. But as is shown in column 3 of Table I, a considerably greater proportion of tumors, thirteen out of seventeen, formed secondary growths, usually in several organs. Thus one frog had numerous metastatic tumors scattered through the liver, the lungs, pancreas, ovary, mesentery, lumbar plexus, and spleen (Figs. 12 to 14). Similarly in another frog there was widespread involvement of liver, lungs, mesentery, and urinary bladder (Figs. 15 to 18). Such extensive involvement has seldom been encountered by us in recently caught frogs or in frogs maintained under more natural conditions of environmental temperature. It seems reasonable to attribute the much higher incidence of metastasis in this over the first series to the healthier condition, and particularly the better state of nutrition, of the animals.

In the twelve well nourished control frogs, freshly acquired and sacrificed during the period of the experiment, none of the tumors had metastasized.

The results in the two series are summarized in Table II. Here we have

combined the groups living at temperatures to which they are accustomed in nature, 7° and 18°, and the groups in which the frogs were exposed to 28°, to which they are not accustomed for long periods. In this combination the nutritional state of the animals has for the present been left out of consideration. The table shows that at 7° and in the control group at 18° only 6 per cent of the tumors have metastasized, whereas at 28° the percentage is increased to nine times this figure, namely 54 per cent. But increase in the occurrence of metastasis is not the only change: there is a much greater degree of dissemina-



TEXT-FIG. 1. Organ distribution of metastases induced by exposing frogs with primary kidney tumors to a temperature of 28°C. The graph is based on nineteen animals. The degree of dissemination is considerably greater than that encountered under more natural conditions of environmental temperature.

tion. The organ distribution of the secondary tumors is graphed in Text-fig. 1, which is based on the nineteen metastasizing tumors included in columns 2 and 3 of Table 1. The liver and the lungs are most frequently involved; and as already emphasized the secondary growths are usually multiple and are frequently extraordinarily numerous. It may be concluded, then, that prolonged elevation of temperature has in some manner facilitated the process of metastasis.

Effect of Temperature on the Primary Tumors.—The mechanism by which elevation of temperature influences metastasis of frog carcinoma is doubtfully complex, but we can analyze in these experiments at least one possible com-

ponent, namely the effect of temperature on the primary tumor. In previous experiments we had found that increase in temperature greatly accelerates the growth of transplants of frog carcinoma (3). Are primary tumors similarly affected? Is there any correlation between change in their size and the occurrence of secondary tumors?

The results obtained in two groups of frogs maintained at 7° and at 28°, and subjected periodically to Roentgen ray examination, are collected in Table III. The findings were contrary to our expectations. Definite enlargement of the kidney tumors was noted in relatively few cases (an example of which is shown in Figs. 6 to 8); in the majority there was no significant change;

TABLE III

Effect of Temperature on Growth of the Primary Tumors; Relation of Change in Their Size to the Occurrence of Metastasis

Temperature	No. of animals	Duration of exposure	Change in size of tumors	Metastasis	
				Present	Absent
28°C. (1st series)	18	days			
		Average: 47	Increase: 5	1	4
		Range: 21 to 84	No change: 9	4	5
			Decrease: 4	1	3
7°C.	21	Average: 98	Increase: 0	0	0
		Range: 58 to 109	No change: 13	2	11
			Decrease: 8	0	8

in some tumors there was actually slight shrinkage (Figs. 3 to 5). Moreover, there was no correlation between change in size of the primary tumors and presence or absence of secondary tumors. Metastasis occurred, or failed to occur, irrespective of whether the primary tumors enlarged, remained stationary, or shrank.

COMMENT

At least three steps are involved in the formation of a metastatic tumor: initially, invasion of tissues (including vascular channels and body cavities); next, transport of detached cells or fragments of tumor, and their mechanical arrest; finally, establishment of intimate contact with a suitable tissue and acquisition of a vascular stroma. For a detailed discussion of these processes the comprehensive texts of Willis should be consulted (1). Here it must suffice to inquire whether there is any indication that temperature influences these sequential events in the frog carcinoma.

Turning first to invasiveness, we had previously observed that at 28° intraocular transplants of frog carcinoma more readily invade the usually

resistant cornea than they do at lower temperature (3). Recently Coman (7) and McCutcheon, Coman, and Moore (8) have brought convincing evidence that invasion of tissue by cancer depends principally upon two factors: (a) decreased mutual adhesiveness of malignant cells which facilitates their separation from each other so that they become detached units, and (b) ameboid movement of the detached cells, whereby they are enabled to wander into the surrounding parts. Both of these factors are influenced by temperature. Regarding mutual adhesion, Zeidman (9) has lately demonstrated that this property is diminished in normal cells by an increase in temperature; although his work has not as yet been extended to malignant cells, it seems fair to assume that they are affected in like manner.

More directly bearing on the subject is the effect of temperature on growth of frog carcinoma in tissue culture (10). This tumor, an adenocarcinoma, exhibits two types of growth with regularity. The first is in the form of tubules which extend out into the semisolid medium and retain their form as long as they are completely enveloped. When, however, the tubules make contact with an unyielding surface, they adhere to it, and the character of growth now changes into a flat membrane. At the margin of the membrane, cells tend to become detached from the growing mass; elevation of temperature not only causes more rapid extension of the membranous growth, but more ready detachment of its marginal cells (11).

The separated cells exhibit active locomotion by means of broad ruffle pseudopodia. A photograph of such a migrating cell is shown in Fig. 12 of an earlier paper (10); the rate of locomotion of these cancer cells as seen in cinematographic films is given in Figs. 13 to 20 of that paper. Thus, in frog carcinoma invasiveness and the principal factors believed to be responsible for it, are definitely affected by temperature.

After the cells have found their way into vascular channels, they are transported by the flowing current until they are finally trapped. The fate of such emboli has been studied by numerous investigators, and there is general agreement that the great majority of the arrested tumor cells do not develop into metastases but are destroyed (12-17). In other words, tumor emboli are not metastases; before they can become such they must survive for a sufficient length of time to multiply and penetrate through the wall of the vessel in which they are mechanically arrested; thereafter all save the most invasive growths must induce the formation of a supporting vascular stroma by the adjacent tissue (12, 18). Now it has been demonstrated that elevation of temperature does favor vascularization of frog carcinoma implanted into alien soil (3). When bits of this tumor are transplanted to the anterior chamber of the eye, vascularization of the implants is invariably more prompt and more efficient at 24° than at lower temperature.

None of these are not the only factors concerned in the metastasizing

effect of temperature on frog carcinoma. It seems quite possible, however, that those mentioned as well as others can be subjected to analysis by further experiments.

SUMMARY

Metastasis of the kidney carcinoma of leopard frogs (*Rana pipiens*) has been induced by exposing tumor-bearing animals for approximately 50 days to a constant temperature of 28°C. Under these conditions 54 per cent of the frogs developed secondary growths, whereas in groups kept at 18° or at 7° metastatic dissemination was found in only 6 per cent. Moreover, at the elevated temperature the metastases were usually more numerous and more widely disseminated; they were also fairly uniform in size, suggesting that they had developed at nearly the same time.

Dissemination of the kidney tumors was influenced by the nutritional state of the frogs, occurring more readily in well nourished than in poorly nourished animals.

Periodic Roentgen ray examinations showed that the size of the primary tumors was not significantly or uniformly affected during the course of the experiments. No correlation was found between change in size of the kidney tumors and the incidence of their metastasis.

Although the mechanism by which temperature induces metastasis of frog carcinoma cannot as yet be elucidated, previous experiments with this tumor indicate that certain factors at least may be involved: Elevation of temperature has been found to cause more ready detachment of cells of frog carcinoma in tissue culture; to bring about increased velocity of locomotion of the detached cells; to lead more promptly and efficiently to vascularization of transplants; and to effect their greater invasiveness.

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EXPLANATION OF PLATES

PLATE 10

FIG. 1. Roentgen ray film showing the appearance of bilateral kidney tumors in a frog whose body cavity had previously been injected with air. (The markings along the margin of the film are at intervals of 1 cm.)

FIG. 2. The kidney tumors as they appear at postmortem examination 1 day after the Roentgen ray film was made. Comparison of Figs. 1 and 2 shows that the size and shape of the tumors can fairly well be determined by Roentgen ray examination after intracelomic injection with air.

FIGS. 3 to 5. A series of Roentgen ray photographs of kidney tumors in a frog which had been kept at 7°C. for a period of 94 days. The three films were made on January 21, March 10, and April 19, respectively; they show moderate and progressive reduction in size of the tumors. The decrease in size of the tumors is associated with nutritional wasting, as can be seen by comparing the outlines of the thighs.

FIGS. 6 and 7. Roentgen ray films of a kidney tumor as seen from the side, made on January 21 and on March 10, respectively. The frog had been kept at a temperature of 28° for 42 days.

Comparison of these films shows a considerable increase in size of the primary tumor, and a metastatic spread upward to the liver and anterior to the parietal peritoneum.

FIG. 8. Postmortem appearance of the same frog 2 days after the last Roentgen ray examination. The figure shows the primary kidney tumor, and the multiple metastases in the liver and on the parietal peritoneum.

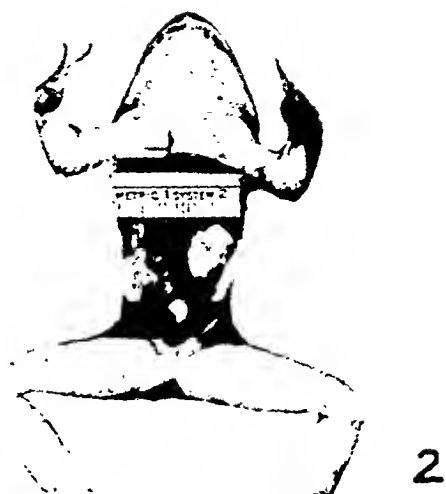


PLATE 11

FIG. 9. A frog which had been kept for 24 days at 28°C. Roentgen films taken before the animal was placed at this temperature revealed no evidence of metastasis. At autopsy the liver and lungs were found riddled with metastatic tumors (see Figs. 10 and 11).

FIG. 10. Representative section of lung from frog shown in Fig. 9. The organ is almost completely replaced by small tumors of fairly uniform size. $\times 30$.

FIG. 11. Section of liver from same frog showing numerous metastases. $\times 30$.

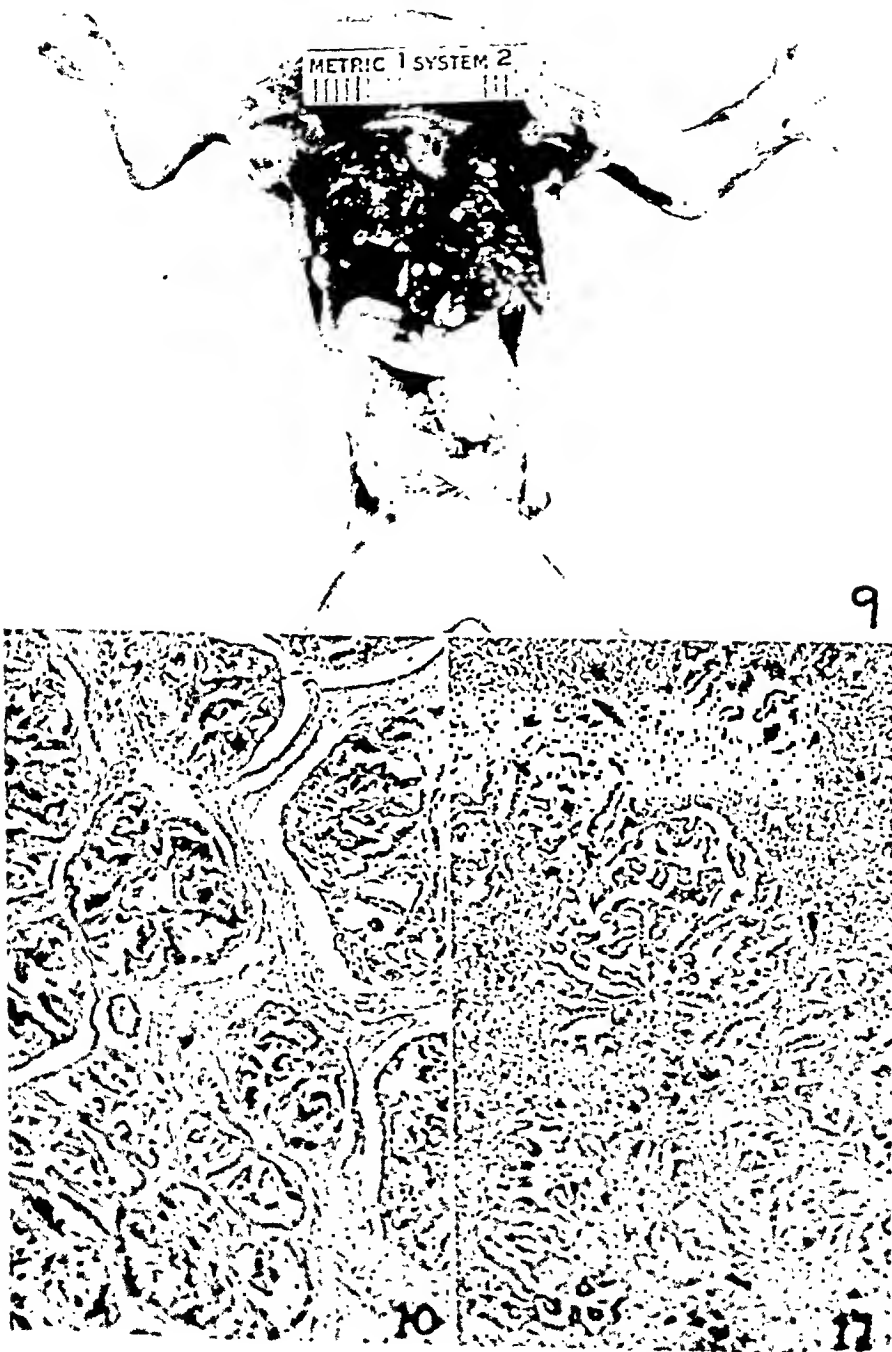


PLATE 12

FIG. 12. Extensive metastasis in frog which had been exposed to 28°C. for 45 days. The photograph shows massive bilateral primary tumors of kidneys, filling nearly the lower half of the celomic cavity. Numerous metastatic tumors are scattered through the liver, involving particularly the right lobe. To the left of the midline, and touching the lower margin of the liver is a metastatic tumor in the ovary (the eggs appear as black dots over the surface of the tumor). Additional metastatic tumors are present in the spleen (Fig. 13), in a lumbar nerve (Fig. 14), in the pancreas, the mesentery, and in the lungs.

FIG. 13. Section of a metastatic tumor in spleen. $\times 30$.

FIG. 14. Metastatic tumor involving and partially destroying a large nerve from the lumbar plexus. $\times 8$.

FIGS. 15 to 18. Multiple metastatic tumors from a frog exposed for 39 days to 28°C.

FIG. 15. Metastatic tumors in the urinary bladder. $\times 3$.

FIG. 16. Metastatic tumors in both lungs. $\times 3$.

FIG. 17. Metastatic tumors in mesentery. $\times 3$.

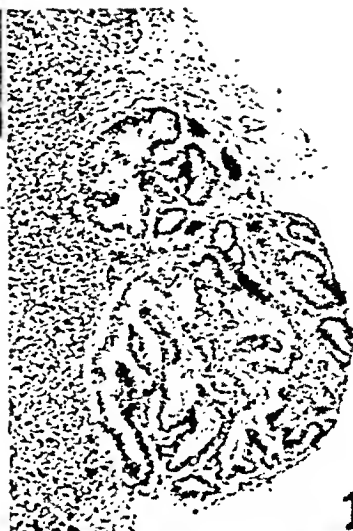
FIG. 18. Metastatic tumors in liver. $\times 3$.



METRIC 1 SYSTEM 2

3

12



13



14



15



17



16



18

ONE-STEP GROWTH CURVES OF VARIOUS STRAINS OF INFLUENZA A AND B VIRUSES AND THEIR INHIBITION BY INACTIVATED VIRUS OF THE HOMOLOGOUS TYPE*

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Studies on the propagation of influenza viruses in the chick embryo (1) have shown that under certain conditions one-step growth curves may be obtained which are similar in principle to those observed in bacterial host-virus systems (2, 3). Upon allantoic injection of either the PR8 strain of influenza A or the Lee strain of influenza B virus, only part of the active virus is adsorbed by the cells lining the allantoic cavity. The amount of residual free virus of the seed, which can be assayed in the allantoic fluid of the injected eggs by infectivity titrations, remains constant for a period of 5 to 6 hours in the case of the PR8 strain, and for 8 to 9 hours in that of the Lee strain. After this constant period, the virus titer increases as a result of liberation of newly formed virus from the infected tissue. Part of the released virus is adsorbed immediately upon some of the remaining uninfected host cells, and the rise in titer in the allantoic fluid is consequently relatively slow. However, if one induces the interference phenomenon in the remaining susceptible cells by injection of large amounts of heterologous virus inactivated by irradiation with ultraviolet light (4, 5), most of the liberated virus remains free in the allantoic fluid and a rather sharp increase in infectivity is noted at the end of the constant period. The release of virus extends over 2 to 4 hours. Thereafter, the infectivity titer of the allantoic fluids remains stationary at the new plateau, since no additional host cells have become infected and consequently no further virus has been propagated.

The use of irradiated heterologous virus for the blockade of the remaining susceptible host cells, as indicated above, permits the study of a single infectious cycle from adsorption of the seed to liberation of the new generations of virus. However, with injection, following infection, of homologous, rather than heterologous, irradiated virus, a marked reduction in the yield of virus was noted. This effect might be due to inhibition of virus production in, or its release from, the host cells. Evidence for the former alternative will be presented elsewhere (6).

In the experiments to be reported, growth curves of several strains each of

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influenza A virus, including one strain of swine influenza virus, were compared with each other and with those of three strains of influenza B virus. It will be shown that all strains of influenza A virus exhibited constant periods of 5 to 6 hours, whereas 8 to 10 hours elapsed before influenza B virus was released from the infected host cells. It also will be demonstrated that the inhibitory effect of the homologous irradiated virus, when injected after infection with active virus, is a type-specific and not strain-specific property. There exist, however, reciprocal quantitative differences in cross-inhibition by irradiated influenza A and swine influenza viruses.

Materials and Methods

Viruses.—The following strains of influenza virus were used: PR8 (7), WS (8), Melbourne (9), F99 (10), and L₇47 (11) of Type A; S15 of swine influenza (12); and Lee (13), ES (14), and Saha (15) of Type B. The methods used for the preparation of seed, the irradiation of virus with ultraviolet light, and the assay of infectivity have been fully described (1). No changes in technic have been introduced except for the use of concentrated irradiated virus preparations for some of the experiments. This concentration was achieved by high speed centrifugation of the infected allantoic fluids at 14,000 r.p.m. for 1 hour, and resuspension of the sedimented virus in a fraction of the supernatant fluid to effect a four- or eightfold concentration. Dialysis of the concentrate and irradiation were carried out in the manner previously described.

Growth Curve Technic.—Adequate numbers of 12-day-old chick embryos were infected by the allantoic route with 1,000 to 10,000 ID₅₀ of virus (0.2 ml. inoculum), and returned to the incubator. One hour later a second injection was given, by the same route, of 0.5 ml. of preparations of homologous or heterologous irradiated virus. Following further incubation at 36–37°C., 5 to 6 eggs of each series were removed at 1 to 2 hourly intervals for harvest of the allantoic fluids. These were collected by means of needle and syringe, without previous chilling of the eggs. The fluids of corresponding eggs were pooled and stored at 4°C. until titrations for infectivity could be made in 10-day-old chick embryos. The results of the titrations are expressed as the number of ID₅₀ per ml. of allantoic fluid.

EXPERIMENTAL

Comparison of Various Strains of Influenza A and B Viruses.—One-step growth curves were obtained with five strains of influenza A and one strain of swine influenza virus. In these experiments, irradiated Lee virus was employed for the blockade of the remaining uninfected cells of the host tissue. For obtaining the growth curves of three strains of influenza B virus, preparations of irradiated influenza A virus (PR8) were injected as the blocking agent 1 hour after infection. The growth curves obtained with the various strains of influenza A and swine influenza virus, on the one hand, and of the three strains of influenza B virus, on the other, resembled each other closely. Since such curves for the PR8 and Lee strains have been published in detail, it suffices here to tabulate the essential findings. As can be seen in Table I, the extent of adsorption of seed virus varied from 63 to 90 per cent. These figures were obtained by determining the amount of virus injected (titration of the seed), and subtracting from these values the quantities of virus found free in the

allantoic fluids of the injected eggs during the constant periods. The difference was considered as the amount of virus adsorbed. The percentage of seed virus thus calculated to be adsorbed varied with individual strains (PR8 or Lee) from test to test over a fairly wide range (42 to 96 per cent), as will be shown elsewhere (16). This variability in all likelihood is caused by the inaccuracies inherent in the methods of assay employed. The variations in the percentage of adsorption observed in the experiments recorded in Table I cannot be interpreted, therefore, as an indication of differences in the various strains used.

The constant periods extended over 5 to 6 hours in the case of all the influenza A and swine influenza strains, and over 8 to 10 hours in the case of the influenza B viruses. It appears from this observation that the constant period is

TABLE I
One-Step Growth Curves with Various Strains of Influenza A and B Virus

Strain	Type	No. of experiments	Seed virus adsorbed	Constant period	Release period	ID ₅₀ virus released per ID ₅₀ adsorbed
			<i>per cent</i>	<i>hrs.</i>	<i>hrs.</i>	
PR8	A	8	71	5-6	2-3	63
WS	A	1	90	6	4	104
Melbourne	A	2	68	5-6	2-3	60
F99	A	2	74	5-6	3-4	77
L47	A	1	72	5	3	48
S15	Swine	3	83	6	3-4	81
Lee	B	7	73	8-9	3-4	36
IS	B	2	63	8-9	3-4	40
Saha	B	1	89	10	3	31

characteristic for each of the two types, but that there exist no marked differences in this respect among the various strains of one type.

The time required for the release of virus from the infected host cells is again somewhat variable from experiment to experiment. Thus, no consistent differences have been discernible between influenza A and B strains, nor within each type.

Finally, the yield of virus, i.e. the number of ID₅₀ liberated per ID₅₀ of seed adsorbed (Y), also varies over a fairly wide range, probably again because of the technical difficulties inherent in the methods of assay of influenza virus. However, it seems quite definite that there exists a difference between the influenza A and B strains, in that the latter always show a distinctly lower yield of virus than the former. It should be pointed out that the amount of virus liberated into the allantoic fluid does not reflect the total virus production (16).

The Inhibitory Effect of Homologous Irradiated Virus.—In an other set of

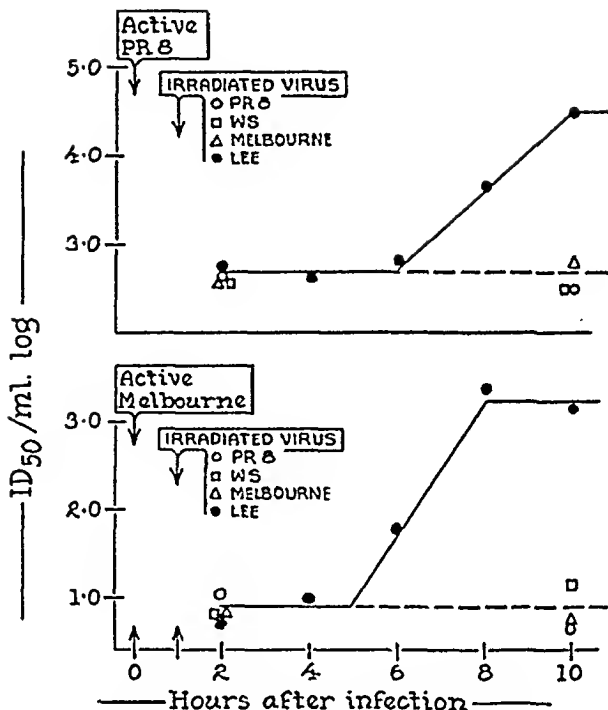


FIG. 1. The inhibitory effect of irradiated virus of various strains of the homologous type on one-step growth curves.

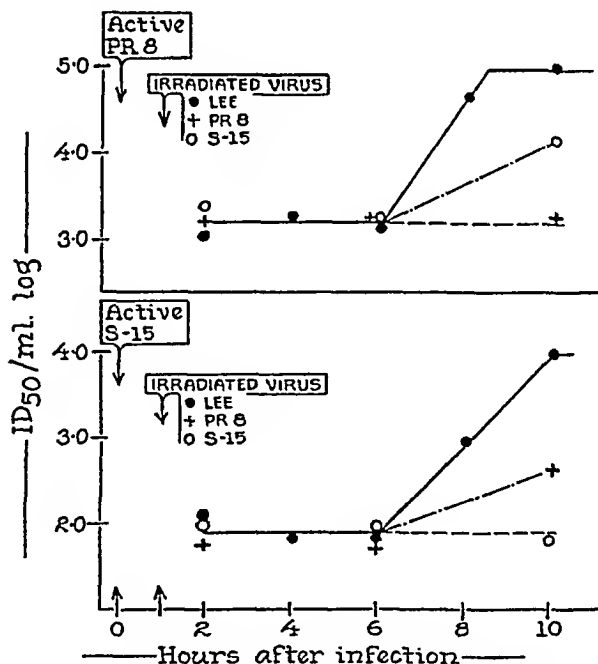


FIG. 2. Partial cross-inhibition of one-step growth curves between irradiated PR8 and S15 strains of influenza virus.

experiments, the inhibitory effect of irradiated virus of the homologous type was studied. In these tests, the blocking agents were prepared from centrifugally concentrated suspensions of virus, since undiluted, inactivated allantoic fluids did not produce complete inhibition of the step with regularity. As can be seen in Fig. 1, the irradiated preparations of the PR8, WS, and Melbourne strains inhibited equally well the appearance of newly formed active virus in the allantoic fluids of the eggs infected with active PR8, Melbourne, or, not shown in the figure, with WS virus. On the other hand, when using irradiated concentrated Lee virus for the blocking, the usual sharp steps in the virus concentration were obtained after constant periods of 5 to 6 hours.

These experiments indicated that the inhibitory effect was type-specific but not strain-specific. In this connection, it was of interest to study whether the behavior of swine influenza virus was like that of human influenza A strains. As shown in Fig. 2, irradiated concentrated S15 virus produced an inhibitory effect in growth curve experiments with active PR8 virus, but to a lesser extent than the irradiated homologous PR8 strain. Conversely, the step in S15 growth curves was completely inhibited by irradiated S15 virus, but only partially by inactivated PR8 virus. Repetition of the experiment again showed only partial cross-inhibition.

DISCUSSION

The data presented reveal that as far as comparison between strains of types A and B is concerned, there exist marked differences in the constant periods; *i.e.*, in the periods in which the virus presumably multiplies in conjunction with the host cells. All influenza A strains studied showed constant periods of 5 to 6 hours, the B strains of 8 to 10 hours. Furthermore, the quantity of virus released into the allantoic fluid for every ID_{50} of seed virus adsorbed was found distinctly larger in the case of influenza A than in that of influenza B virus. No consistent differences are apparent in comparing the constant periods of various strains of one type. It must be emphasized, however, that the methods employed for the quantitative harvest of the allantoic fluids and the assay of virus activity are relatively inaccurate, and that the time intervals chosen for testing are rather widely spaced. It is possible, therefore, that smaller differences in the constant periods among various strains of one type may well have escaped detection.

tory showed that passage through eggs of a mixture of four strains of influenza A virus (PR8, WS, Melbourne, and F99) resulted after three transfers in a culture which contained, as far as demonstrable, only the Melbourne strain, in spite of the fact that the constant periods of these strains extend over similar periods of time. It was felt that a slight numerical advantage of one strain in the mixed seed used for the first passage might have led to increasingly greater advantages in the subsequent passages, so that the other strains were gradually prevented from producing sufficient concentrations of virus to become demonstrable by the hemagglutination test. It is possible, particularly in the light of the apparent simultaneous passage of influenza A and B strains (18), that factors other than those discussed may determine the survival or loss of strains in mixed infections.

It has long been apparent that there exist certain cross-relationships between the human influenza A and swine influenza viruses. Common antigenic components among these agents have been demonstrated in neutralization and immunization tests (19-21), by inhibition of hemagglutination (22, 23), and the soluble complement-fixation antigens of the two groups of viruses appear indistinguishable by the methods employed (24, 25). It was not surprising, therefore, that the growth curve experiments failed to reveal marked differences between influenza A and swine influenza viruses. Cross-inhibition of virus propagation by injection, following infection, of irradiated virus likewise indicated a close relationship. However, in this case, the inhibitory effect upon propagation of the PR8 strain was more pronounced with the homologous irradiated PR8 virus than with the irradiated S15, and conversely. The strains of influenza A virus, on the other hand, appeared to be identical in this respect.

SUMMARY

One-step growth curves of five strains of influenza A, one strain of swine influenza, and three strains of influenza B virus have been analyzed.

The influenza A and swine influenza strains showed constant periods of 5 to 6 hours before newly formed virus was liberated from the infected cells, whereas 8 to 10 hours elapsed in the case of the influenza B strains.

The yield of virus in the allantoic fluids, *i.e.* the number of ID₅₀ released for every ID₅₀ of seed virus adsorbed, was consistently higher in the case of the influenza A and swine influenza strains than in that of the influenza B viruses.

Interruption of the cycle by injection of inactivated virus subsequent to infection can be achieved by any of the strains of the homologous type. However, cross-tests between influenza A and swine influenza virus led only to partial inhibition of virus growth.

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OBSERVATIONS ON THE PREGNANT RAT INJECTED WITH NEPHROTOXIC RABBIT ANTI-RAT PLACENTA SERUM AND DESOXYCORTICOSTERONE ACETATE*

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In a previous report (1) Seegal and Loeb have described a series of experiments designed to test the idea that a toxemia of pregnancy may result from the action of injurious antibodies evoked by antigenic substances of placental origin. The injection of rabbit anti-rat placenta serum into pregnant rats regularly produced abortion, and, when these animals were observed over a subsequent period of 3 to 14 months, over 50 per cent of them developed chronic glomerulonephritis. However, since non-pregnant females, as well as males, given this serum developed nephritis with equal frequency, the chronic renal lesions could not be considered a manifestation of "toxemia of pregnancy," nor were any acute episodes suggesting this condition observed in the animals.

Subsequent observations by Knowlton *et al.* (2) demonstrated that the administration of desoxycorticosterone acetate (DCA) in the presence of a liberal NaCl intake enhances the renal hypertrophy and nephritis produced in rats by another cytotoxic serum; namely, rabbit anti-rat kidney serum. In addition, the animals so treated developed striking hypertension. In view of the fact that in the work of Seegal and Loeb (1), referred to above, no demonstrable effect of anti-placenta serum, other than abortion, occurred during gestation, the present experiments were planned to determine whether a toxemia of pregnancy might result if the serum were fortified by DCA and NaCl.

EXPERIMENTAL

Fifty-two female rats of the Long Evans strain ranging from 52 to 75 days of age were raised on the following basic diet:

Whole wheat flour	65.8 per cent
Careon	26.0 " "
Wesson oil or butter	4.0 " "
Cod Liver oil	2.9 " "
CaCl ₂	1.5 " "
NaCl	1.6 " "

* Supported by grants from the United States Public Health Service and the Albert and Mary Lasker Foundation.

The rats were divided into six groups (Table I). Groups I, II, and III were maintained on this basic diet. For groups IV, V, and VI the diet was adjusted to contain 1.5 per cent NaCl and 0.2 per cent NaCl was added to the drinking water in order to enhance the action of the desoxycorticosterone acetate (DCA)¹ which these groups received. Two and one-half mg. of DCA, suspended in peanut oil, 10 mg./cc., were injected daily subcutaneously in different areas over the back throughout the experiment beginning with the 1st day of each rat's gestation period in groups IV and V and throughout equal periods in the non-pregnant animals in group VI. The animals had been bred at estrus and vaginal smears were examined for spermatozoa. One-half of the individuals in each group were sacrificed after 3 weeks of observation. This corresponded to the calculated last day of gestation in the pregnant animals. The remaining half of the animals in each group were observed over a 7 week period.

The rabbit anti-rat placenta serum used was a pool made up of equal amounts of serum obtained from two rabbits immunized according to the method previously described (1). The serum, inactivated by heating to 56°C. for 20 minutes, was given intravenously in doses of 0.4 cc., 0.5 cc., and 0.6 cc., respectively, on 3 successive days 9 days after the initial DCA injection. In pregnant animals, this corresponded to the 9th day of gestation.

TABLE I
Distribution and Treatment of Animals

Group	No. of rats	Condition	NaCl in diet	NaCl in drinking water	DCA	AP serum*
			per cent	per cent	mg. per day	cc.
I	12	Pregnant	1	0	0	0
II	12	Pregnant	1	0	0	1.5
III	6	Not pregnant	1	0	0	1.5
IV	8	Pregnant	1.5	0.2	2.5	0
V	14	Pregnant	1.5	0.2	2.5	1.5
VI	12	Not pregnant	1.5	0.2	2.5	1.5

* AP serum = rabbit anti-rat placenta serum.

Systolic blood pressure measurements, according to the modification of the method of Williams, Harrison, and Grollman (3) described by Sobin (4) were made on all animals except those in groups I and III. Two or three preliminary readings at weekly intervals were made on each animal. Thereafter, weekly readings were recorded throughout the course of the experiment. Each reading is an average of ten consecutive observations. Fig. 1 presents the average of the weekly readings of all animals in groups II, IV, V, and VI as a heavy line, while the weekly individual extremes appear in the upper and lower broken lines.

Preliminary qualitative albumin determinations by the heat and acetic acid method were done on all animals. A quantitative determination of urinary albumin, using the technique of Shevky and Stafford (5), was made on random samples the day before the calculated date of delivery in all pregnant animals sacrificed at term with the exception of those in group I, as well as at a corresponding period after the administration of serum in the non-pregnant rats of group VI. An additional determination of urinary albumin was done at the termination of the experiment in those animals observed over a longer period of time.

Blood was withdrawn from the heart for the determination of urea nitrogen by the micro-

¹ The authors wish to acknowledge their indebtedness to Dr. Kenneth Thompson of Roche Organon, Inc., Nutley, New Jersey, for supplying the DCA used in these experiments.

Kjeldahl technique at the termination of the experiment, when the animals were weighed and sacrificed with ether. The uterus was examined for degenerating placentae, placental sites, or viable young. The heart, kidneys, and adrenals were weighed and sections removed for histological study. Sections of lungs, liver, spleen, and ovaries were also examined microscopically.

RESULTS

Abortion.—Abortion occurred in 100 per cent of pregnant animals given anti-placenta serum. The placentae had been completely resorbed or extruded in these animals examined at term. Placental sites were noted as proof that the animals had been pregnant. No instance of abortion occurred among the un-

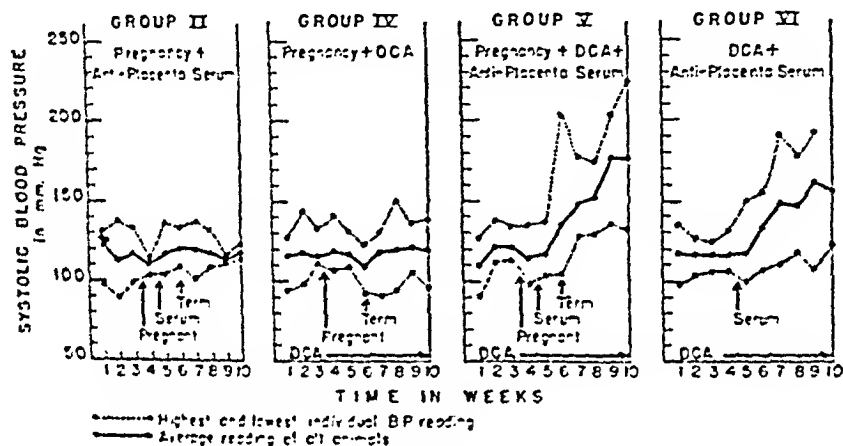


FIG. 1. The results of weekly blood pressure determinations on rats in designated groups.

treated pregnant animals of group I. One pregnant rat of group IV treated with DCA alone aborted.

Blood Pressure.—Fig. 1 presents the systolic blood pressure readings of all animals in groups II, IV, V, and VI. No determinations were made on rats in groups I and III. Hypertension occurred only in those animals receiving DCA in addition to rabbit anti-rat placenta serum; i.e., groups V and VI. A definite trend toward the development of hypertension is apparent at the time of the calculated date of delivery in group V and after a similar time interval among animals in group VI. Among the 7 animals of group V and the 6 animals of group VI, where observations were continued over an additional 4 week period, the blood pressure continued to rise, the average reading being slightly higher in the group V animals. Whether this is a significant difference, in view of the small number of rats observed, is questionable.

Albuminuria.—As has been previously stated, no albumin determinations were done in group I. In the remaining groups the results of the qualitative

determination of urinary albumin at the beginning of the experiment varied from 0 to ++, which is normal for this strain of rats. The results of quanti-

TABLE II

Compilation of Significant Data: Body and Organ Weights; Distribution and Extent of Nephritic Lesions; Quantitative Urine Albumin

	Group I		Group II		Group III		Group IV		Group V		Group VI	
	Pregnant		AP serum* Pregnant		AP serum* Non-pregnant		Pregnant DCA		AP serum* Pregnant DCA		AP serum* Non-pregnant DCA	
Duration of experiment, wks.....	3‡	7	3‡	7	3‡	7	3‡	7	3‡	7	3‡	7
No. of animals..	6	6	6	6	3	3	4	4	7	7	6	6
Average body weight, gm....	188	190	179	194	168	190	165	198	170	194	163	184
Range, gm.....	160-220	170-220	150-200	165-205	160-180	180-200	150-173	175-210	150-185	170-215	135-190	162-203
Average heart weight, mg....	629	618	628	674	643	646	610	780	694	930	690	834
Range, mg.....	587-712	524-705	501-709	646-782	629-663	559-711	563-694	653-897	583-819	795-1186	563-823	679-957
Average adrenal weight, mg....	50	46	54	49	60	47	47	43	51	42	42	40
Range, mg.....	44-57	38-53	44-68	43-61	51-67	44-52	40-52	35-48	47-63	35-50	35-52	30-47
Average kidney weight, mg....	1191	1232	1503	1415	1169	1285	1572	1774	2142	2054	1721	1917
Range, mg.....	1128-1308	1129-1397	1168-2035	1211-1716	1156-1197	1242-1366	1391-1785	1562-2150	1624-2970	1781-2303	1400-2005	1465-2307
Quantitative urine albumin, average gm. per cent.....	—	—	3.2	0.8	1.1	0.6	0.1‡	0.1	4.0	0.5	1.8	0.6
Range, gm:.....			0.4-5.9	0.2-1.9	0.6-1.7	0.3-0.9	0.1-0.1	0-0.2	1.0-7.2	0.1-1.0	1.0-4.7	0.3-1.1
Intensity 0	IIIIII	IIIIII	III	IIII	III	III	IIII	IIII	II	—	III	III
and dis- +	—	—	II	I	—	—	—	—	III	II	II	II
tribu- ++	—	—	I	—	—	—	—	—	I	—	I	I
tion +++	—	—	—	—	—	—	—	—	I	—	—	—
of +++++	—	—	—	—	—	—	—	—	—	III	—	—
nephritic lesions										I		

* Rabbit anti-rat-placenta serum.

‡ End of gestation period, or at an equivalent point in time in non-pregnant animals.

§ Average of 3 rats; see text.

|| Each "I" = 1 animal.

tative albumin determinations are given in Table II. It will be seen that the group IV rats, which received DCA but no cytotoxic serum, continued to have normal amounts of urinary albumin when quantitative determinations were made at term as well as 4 weeks later, with one exception. This animal pre-

sented significant albuminuria which was, however, unassociated with histological evidence of renal pathology and this value is omitted from the table. On the other hand, all serum-injected animals, *i.e.* those of groups II, III, V, and VI were observed to have significant albuminuria when examined at "term," 9 days after serum administration. This was far in excess of the amount attributable to rabbit protein in the injected immune serum and was conspicuously more marked in serum-treated pregnant animals both with and without DCA (groups V and II, respectively) than in the similarly treated non-pregnant animals (groups VI and III, respectively). The heavy albuminuria observed in serum-treated animals decreased in intensity and subsequent determinations made at 7 weeks showed consistently lower values. A comparison was made between the lesions seen histologically and the extent of the albuminuria in individual animals and no consistent correlation between the two could be found.

Blood Urea Nitrogen.—With two exceptions, the values for blood urea nitrogen were normal for all animals in each group. One value of 37 mg./100 cc. occurred in an animal of group IV and remains unexplained. The other was a value of 39 mg./100 cc. in an animal of group V which was found at autopsy to have nephritis and generalized arteritis.

Organ Weights.—Body and organ weights are presented in Table II and it will be seen that the body weights are comparable in all six groups.

Progressive cardiac enlargement had occurred in all groups receiving DCA, *i.e.* groups IV, V, and VI, when observed at the end of a 7 week period. As can be seen in the table, this was greatest in the hypertensive animals of groups V and VI, which received nephrotoxic antiplacenta serum in addition to daily DCA administration. The difference in magnitude between these two groups is of doubtful significance. These findings parallel previous observations (2) on the cardiac hypertrophy occurring in non-pregnant female rats as well as in male rats treated with DCA or with DCA plus anti-kidney serum. The cardiac enlargement in animals receiving serum unfortified by DCA, *i.e.* groups II and III, is of questionable significance.

The observed adrenal weights (Table II) suggest that DCA administration may have partially counteracted the anticipated hypertrophy due to pregnancy.

The administration of anti-placenta serum by itself did not result in renal enlargement during the period of observation (see group III in Table II), the weights recorded here being within the normal range for rats of this age, sex, and strain (unpublished data). However, the injection of anti-placenta serum in pregnant rats was followed by enlargement of the kidneys during gestation (see group II, Table II).

The addition of administration of DCA with adequate NaCl (see groups IV, V, and VI, Table II) resulted in marked renal enlargement in all animals. As in the groups which received no other treatment, the enlargement was more

striking in those rats given anti-placenta serum during pregnancy (group V, Table II).

Histopathology.—Both kidneys of each animal were examined histologically and the presence of cytotoxic serum nephritis was graded, as in previous studies (2), 1 to 4 plus according to the extent and severity of the lesions. The results can be seen in Table II. Animals receiving no cytotoxic serum, groups I and IV, had no evidence of nephritis. Of the remaining groups, all of which were serum-treated, 4 instances of nephritis occurred among the 12 pregnant rats of group II in contrast to the absence of renal lesions in all 6 non-pregnant rats of group III. Similarly, when the action of the serum was reinforced by DCA, 12 of 14 pregnant rats of group V developed nephritis in contrast to 6 instances of nephritis among 12 non-pregnant rats of group VI. Moreover the more severe lesions occurred in the former group.

One pregnant rat in group V whose blood pressure reached 204 mm. Hg showed, in addition to marked nephritis, a generalized arteritis at autopsy. All animals receiving DCA showed the previously described (2) tubular lesions characteristic of the action of this steroid in the dosage employed.

All other organs examined showed no significant abnormalities.

COMMENT

Previous studies (1) have demonstrated that rabbit anti-rat-placenta serum injected into rats produces a chronic progressive nephritis that is ultimately indistinguishable from that induced by the injection of specific rabbit anti-rat-kidney serum. Subsequent observations (2, 6, 7) have established the fact that renal and cardiac hypertrophy occur in rats maintained on a liberal NaCl intake and injected daily with DCA. It has also been shown that when this regimen is imposed upon rats whose kidneys have been previously injured with anti-kidney serum both the cardiac and renal hypertrophy are increased, the nephritic process is intensified, and the rats become strikingly hypertensive (2).

The present results confirm the fortifying effect of DCA upon the action of cytotoxic serum, in this instance, anti-placenta serum. In addition, evidence is presented which indicates that the kidney of the pregnant rat is more susceptible than is that of the non-pregnant rat to injury through the action of nephrotoxic anti-placenta serum. This is demonstrated by the higher incidence as well as greater intensity of renal lesions occurring among the serum-treated pregnant animals of groups II and V than among the similarly treated non-pregnant rats of groups III and VI. Thus, it seems probable that pregnancy itself, like DCA, increases the vulnerability of the rat's kidney to injury with anti-placenta serum. Hypertension, which was observed in pregnant animals on adequate NaCl intake, treated with DCA and anti-placenta serum, is not dependent upon pregnancy, inasmuch as a commensurate rise in blood

pressure was observed in non-pregnant animals similarly treated. It appears, furthermore, that the cardiac hypertrophy described is independent of pregnancy.

The question arises whether the effects produced by DCA and anti-placenta serum in pregnant rats constitute a toxemia of pregnancy. The animals aborted, developed hypertension, and renal damage. However, unlike the generally beneficial effects of emptying the uterus in cases of human toxemia, no decline in hypertension occurred following abortion and the renal lesions progressed. For this reason the observed increased susceptibility of the kidney of the pregnant rat to the action of nephrotoxic serum and DCA is perhaps more analogous to the acceleration of chronic nephritis in the human being, which is frequently observed during or following intercurrent pregnancy.

CONCLUSIONS

1. Pregnancy enhances the susceptibility of the rat to intercurrent renal damage produced by anti-placenta serum. This is manifested by the development of renal hypertrophy and nephritis in a number of these animals. Both renal hypertrophy and nephritis are consistently intensified by the concomitant administration of DCA.

2. Hypertension develops in both pregnant and non-pregnant rats treated with the anti-placenta serum employed together with the daily administration of DCA.

3. Termination of pregnancy, in the face of continued DCA administration, fails to lower the blood pressure or to arrest the nephritic process.

We are indebted to Miss Judith Berg for technical assistance.

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QUANTITATIVE STUDIES ON THE TOTAL PLASMIN AND THE TRYPSIN INHIBITOR OF HUMAN BLOOD SERUM

I. METHODS FOR THE TITRATION OF TOTAL PLASMIN AND OF TRYPSIN INHIBITOR

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In the following work an attempt has been made to evaluate quantitatively the amounts of proteolytic enzyme and of trypsin inhibitor in patients' blood before, during, and after the onset of acute rheumatic fever. The terminology suggested by Christensen and MacLeod (1945) has been adopted. Thus active serum proteinase is referred to as "plasmin;" the inactive precursor of plasmin is designated "plasminogen;" the activator of plasminogen is called "streptokinase" (formerly known as streptococcal fibrinolysin), and the specific antibody against the activator is termed "antistreptokinase." Christensen and MacLeod (1945) found that the proteolytic activity of plasmin is inhibited by both the crystalline pancreatic inhibitor of trypsin and by the trypsin inhibitor which is found in serum. They also showed that both these inhibitors are less active against plasmin than against crystalline trypsin and that the different shapes of inhibition curves for the two enzymes suggest that there are also qualitative differences between the inhibition of trypsin and of plasmin. In this paper the inhibitor of trypsin and plasmin which is present in serum is referred to as "trypsin inhibitor" or "inhibitor." The expression "total plasmin" is used to denote the proteolytic activity of serum in which the plasminogen has been activated by an excess of streptokinase. The total plasmin level therefore comprises the sum of both spontaneously activated and streptokinase-activated plasminogen.

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Materials and Methods

The majority of the sera used in this investigation were obtained during March and April, 1946, from scarlet fever patients at the Great Lakes Naval Training Center.¹ The patients were all young men of the same age group. Blood samples were taken on admission to the hospital and thereafter at weekly intervals until the patients returned to duty. Specimens of serum were therefore available from scarlet fever patients who did not develop any complications and returned to duty after about 3 weeks in the hospital, from those who developed otitis media or other purulent complications and remained in the hospital for longer periods, and finally, from those who developed rheumatic fever and received prolonged hospital treatment. The sera were stored in the cold at about 4°C. without any added preservative in sterile tubes with rubber stoppers. They supplied a series of samples in which the total potential proteolytic activity of the serum before the onset of rheumatic fever could be compared with the proteolytic activity at the time of rheumatic onset and during subsequent weeks. The sera from uncomplicated cases of scarlet fever and those from patients with purulent complications supplied controls for comparison with rheumatic fever sera.

Substrate for Digestion Experiments.—Azocoll² was used as the substrate in all the experiments described in this paper. This substance was originally used by Oakley, Warrack, and van Heyningen (1946) for titrating collagenase in filtrates from cultures of *Clostridium welchii*. It is a reddish purple insoluble powder prepared by coupling commercial "hide powder," which is a rich source of collagen, with an azodye. When incubated with collagenase a deep red color is released into the suspending fluid by disintegration of the azocoll. A similar release of color occurs when azocoll is incubated with plasmin or with trypsin, chymotrypsin, pepsin, papain, and streptococcal proteinase. Different preparations of azocoll are not all digested to the same extent by the same amount of enzyme. It is therefore important to use a single preparation for all observations if comparable results are desired.

The following experiment was set up to determine the most suitable weight of substrate to use in digestion experiments. A constant weight of 0.00025 mg. of crystalline trypsin was used to digest varying quantities of azocoll ranging from 20 to 30 mg. with a variation of 1 mg. per tube. It was found that 20 to 25 mg. of azocoll gave a gradually increasing number of color units. On the other hand 25 to 30 mg. of azocoll gave constant readings in color units. It was therefore considered that 30 mg. of azocoll supplied an adequate excess of substrate and this quantity was used in all experiments.

Streptokinase.—A single preparation of streptokinase designated S. K. A. I.³ was used in all the experiments described in this paper. This was prepared by a method similar to that described by Anderson, Kunkel, and McCarty (1948). A group A strain, H105, was grown in neopeptone dialysate broth with the addition of sterile glucose solution and 5 N NaOH as required. The streptococci were removed by centrifugation. The streptokinase in the super-

¹ The author is indebted to Lieutenant Commander John D. Seal, Medical Corps, United States Navy, and the personnel of the United States Naval Medical Research Unit No. 4 for collection of sera and cultures and for supplying the clinical data on the patients. Dr. Robert F. Watson and Dr. Rebecca C. Lancefield represented the Hospital of The Rockefeller Institute for Medical Research in setting up this cooperative project. The author wishes to thank Dr. Homer F. Swift for organizing the joint program which made available the serum and cultures used in this investigation.

² The author is greatly indebted to Dr. Walther F. Goebel for the preparation of azocoll in sufficient quantity to allow the same lot to be used in all the experiments required for this investigation.

³ Streptokinase (lot S.K.A.I.) was kindly supplied by Dr. Maclyn McCarty in sufficient quantity to allow the same lot to be used in all the experiments required for this investigation.

natant fluid was concentrated and partially purified by acid precipitation at pH 3.8 and by ammonium sulfate fractionation. The final product was lyophilized and preserved in the dry state. Experiments, which will be described in a later section of this paper, were carried out to determine the optimal quantity of this preparation of streptokinase (S. K. A. I.) for activation of plasminogen. Thereafter, a standard quantity of S. K. A. I. was used in all experiments in order to obtain comparable results.

Trypsin.—A single lot of crystalline trypsin⁴ was used in all experiments.

Trypsin Inhibitors.—Bovine serum trypsin inhibitor was preserved in dried form.⁵ The soya bean inhibitor of trypsin was preserved in crystalline form.⁶

Antistreptokinase Determinations.—The titrations of sera for antistreptokinase were carried out by the method described by Anderson, Kunkel, and McCarty (1948).⁷

General Method for Estimation of Proteolytic Activity.—The proteolytic activity of the enzyme systems under investigation is determined by measuring the amount of color released from a constant weight of azocoll after incubation for 30 minutes in a water bath at 37°C. Immediately after incubation the tests are transferred to an ice water bath at about 4°C. for 15 minutes to reduce proteolysis to a minimum; they are then filtered through 7½ cm. filter papers⁸ to remove the residue of insoluble substrate. The resulting colored filtrates are transferred to 12 × 75 mm. cuvettes. The per cent transmittance of light with a wave length of 525 mμ is then read on a Coleman Junior spectrophotometer (Model 6A). This is the optimal wave length for demonstrating differences in the color of azocoll digests. The per cent transmittance of light is finally converted into arbitrary color units which are directly proportional to the proteolytic activity of the enzyme under test. A straight line is obtained when the per cent transmittance of light for serial dilutions of azocoll digest filtrate is plotted on semilog paper against color units constructed by assigning a value of 100 units to undiluted digest. This curve is used to convert the per cent transmittance of light into color units in all subsequent experiments. The relation of these color units to the proteolytic activity of different weights of crystalline trypsin can also be obtained by reference to a curve constructed by plotting weights of trypsin against the color units released by the different quantities of trypsin from 30 mg. of azocoll under the conditions described above.

0.15 phosphate buffer at pH 7.8 mixed with an equal volume of normal saline is used to dilute the reagents and to suspend the substrate. All glassware and rubber stoppers are autoclaved before use.

The Titration of Plasmin in Serum.—An attempt was made to titrate the total plasmin in serum after activation of plasminogen by streptokinase. There are a number of difficulties in this procedure requiring special precautions. The activity of the plasmin may be diminished by trypsin inhibitor in the serum, by inhibitor in the streptokinase, by antistreptokinase in the patient's serum (if insufficient streptokinase is added to the test), or by an absolute fall in the level of plasminogen and plasmin in the patient's serum. The following method was adopted for titration of total plasmin. Pyrex glass tubes labeled 13 × 100 mm. by the manufacturers are measured with calipers and those with internal diameters ranging from 10.9 to 11.0 mm. are selected and set up in a test tube rack. To each tube 4.0 cc. of water is added and those which

⁴ The crystalline trypsin was prepared by Dr. Madlyn McCarty.

⁵ The inhibitor was prepared by Dr. E. C. Loomis and supplied through the kindness of Dr. G. Uggas.

⁶ The soya bean inhibitor of trypsin was prepared by Dr. M. Kunitz and supplied through the kindness of Dr. L. R. Christensen.

⁷ The method is greatly indebted to Dr. H. C. Anderson and Dr. Madlyn McCarty who have generously allowed the use of their antistreptokinase serum inactivation in this paper.

⁸ Whatman No. 541 and Whatman Filter papers S and S No. 542.

show the same water level are selected for use as "digestion tubes." In carrying out tests for total plasmin the selected tubes are immersed in an ice water bath. They then receive 2.6 cc. of buffer-saline at pH 7.8, 0.4 cc. of the undiluted serum to be tested, 1.0 cc. of a solution of streptokinase in suitable concentration, and 30 mg. of azocoll. The tubes are closed with rubber stoppers and shaken until the azocoll is thoroughly wetted and dispersed throughout the fluid. They are then transferred to a water bath at 37°C. for 30 minutes during which time they are inverted several times at intervals of 5 minutes to promote contact between the enzyme and the insoluble substrate. After 30 minutes at 37°C. the tubes are transferred to an ice water bath for 15 minutes. Each tube is then inverted several times to mix the contents and the suspension is immediately filtered through paper. The resulting colored filtrates supply the material for estimation of color units as already described. A serum of known plasmin activity is included in each set of titrations.

TABLE I
Titration of Trypsin Inhibitor in Serum

No. of test or control	Volume of buffer-saline	Volume of serum diluted 1/5,000	Volume of trypsin 0.00025 mg./cc.	15 min. in 37°C. water bath	5 min. in ice water bath	Azo-coll mg.	30 min. at 37°C. with inversion every 5 min.	15 min. in ice water bath	Filtered through paper
Test 1	2.2	0.8	1.0			30			
" 2	2.2	0.8	1.0			30			
" 3	2.2	0.8	1.0			30			
Control 1	3.0	0.0	1.0			30			
" 2	3.0	0.0	1.0			30			
" 3	3.0	0.0	1.0			30			
" 4	4.0	0.0	0.0			30			

The Titration of Trypsin Inhibitor in Serum.—A solution of 10 mg. of crystalline trypsin is made in 2.0 cc. of distilled water. Volumes of 0.1 cc. of this solution are pipetted into small 10 × 75 mm. tubes with 0.2 cc. pipettes.⁹ The tubes are then closed by rubber stoppers with sleeve caps and stored at -28°C. On the day of the test the contents of one of these tubes is allowed to thaw and 1.9 cc. of buffer-saline is then added and mixed, making a solution of 0.25 mg. of trypsin per cc. A volume of 0.1 cc. of this solution is measured with a 0.2 cc. pipette into a volumetric flask and the final volume of the trypsin solution is made up with buffer-saline to 100 cc. containing 0.00025 mg. per cc. These tests are then set up in pyrex digestion tubes, of equal diameter by adding to each tube 2.2 cc. of buffer-saline, 0.8 cc. of serum diluted 1/5,000, and 1.0 cc. of trypsin solution containing 0.00025 mg. of crystalline trypsin. They are mixed and incubated in a water bath at 37°C. for 15 minutes to allow time for combination of trypsin and inhibitor. They are then transferred to a chilled water bath for 5 minutes; and 30 mg. of azocoll is added to each tube. The tubes are then stoppered, shaken, incubated at 37°C. for 30 minutes, with inversions every 5 minutes, immersed for 15 minutes in ice water, and filtered through paper as previously described. The serum dilutions are made with 0.2 cc. pipettes as follows: 0.1 cc. undiluted serum + 4.9 cc. buffer-saline = 1/50; 0.1 cc. of 1/50 serum + 9.9 cc. of buffer-saline = 1/5,000. The tests are then set up as shown in Table I.

In Table I tests 1, 2, and 3 represent three different sera under test. Controls 1, 2, and 3 are three separate determinations of the proteolytic activity of 0.00025 mg. of trypsin alone

⁹ Kimble brand serological pipettes. EXAX 0.2 ml. in 0.01 ml.

without any serum; these are included in every set of titrations and their average value is used to calculate the per cent inhibition of tryptic digestion by each serum under test. Control 4, which is also included in each set of titrations, gives the amount of color which escapes from 30 mg. azocoll into 4.0 cc. of buffer-saline at pH 7.8 in 30 minutes at 37°C. This solution is used as the reference (T per cent = 100) when reading the results of tests in the spectrophotometer. It gives a fairly constant reading of about 80 per cent transmittance with this particular lot of azocoll when buffer-saline is used as the reference. There is, however, considerable variation in this respect between different preparations of azocoll.

Additional control tubes containing diluted serum without any trypsin were included in the earlier titrations. It will be demonstrated in a later section of this paper that these serum controls were unnecessary; they were therefore omitted in later titrations.

EXPERIMENTAL RESULTS

Determination of Optimal Time of Incubation for Digestion Experiments

The following experiment was carried out to determine the optimal time of incubation to avoid accumulation of digestion products which might inhibit proteolysis by plasmin.

Five digestion tubes were set up each containing 2.6 cc. of buffer-saline, 0.4 cc. of human serum, 1.0 cc. of streptokinase, in suitable dilution, and 30 mg. of azocoll. The tubes were incubated at 37°C. in the usual way; determinations of the number of color units liberated from the azocoll were made every 15 minutes for 75 minutes. The result of this experiment is shown in Fig. 1.

It will be seen from Fig. 1 that a linear curve was obtained up to 30 minutes. A standard incubation time of 30 minutes was therefore adopted in all experiments.

The Effect of Trypsin Inhibitor in Serum on the Estimation of Total Plasmin

The degree of inhibition of plasmin activity by serum inhibitor is largely dependent on the time allowed for combination of enzyme and inhibitor at 37°C. before the addition of substrate. No combining time is therefore allowed in the method already described for the estimation of total plasmin; and the reagents are kept cold during the time required for pipetting and mixing. The following experiments were carried out to investigate the results of these attempts to minimize the action of serum inhibitor on plasmin activity.

The results of titrating separately the total plasmin and the trypsin inhibitor in samples of serum taken at intervals from the same patient indicate that under the conditions of the tests there is not a reciprocal relationship between the readings obtained for total plasmin and for trypsin inhibitor. In other words, an increase of inhibitor is not necessarily accompanied by a decrease of total plasmin. Conversely a fall in inhibitor level frequently coincides with a similar fall of total plasmin. This is shown in Fig. 2 which gives the results of total plasmin and of inhibitor titrations in sera taken at intervals from a patient with uncomplicated scarlet fever.

It will be seen that the curves for total plasmin and for trypsin inhibitor are of similar shape in Fig. 2.

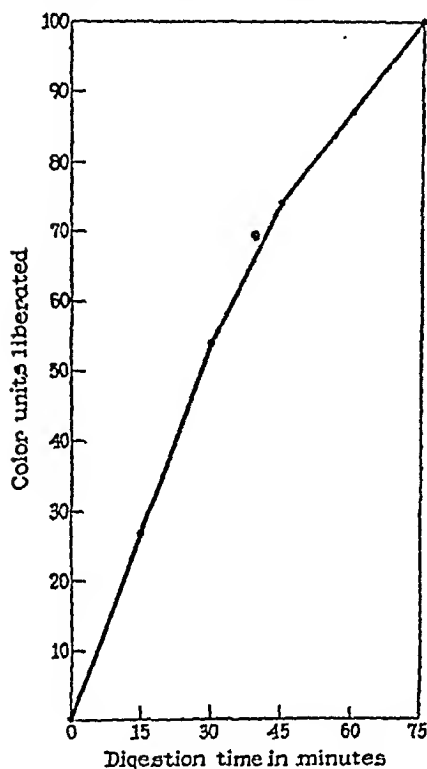


FIG. 1

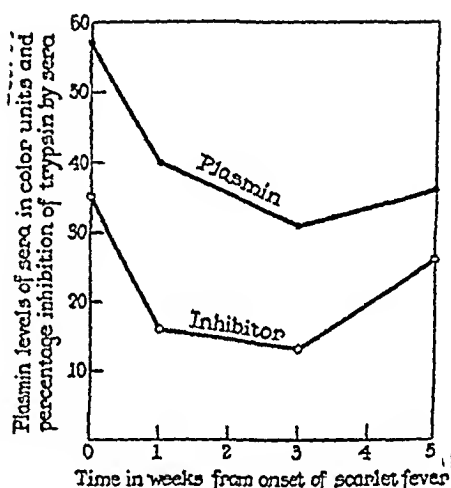


FIG. 2

FIG. 1. Determination of optimal time of incubation for digestion experiments. Abscissae give time of incubation in minutes. Ordinates give color units liberated from 30 mg. of azocoll.

FIG. 2. Results of titrating the total plasmin and the trypsin inhibitor in sera taken at intervals from a patient with uncomplicated scarlet fever. The ordinates give the plasmin levels of the sera expressed as color units recorded thus ●—● and the percentage inhibition of trypsin by the sera recorded thus ○—○. The curves have similar shapes.

These results seemed to indicate that the methods employed were satisfactory for the separate determinations of total plasmin and of trypsin inhibitor. The following experiment was carried out to obtain more direct evidence as to the effect of trypsin inhibitor on the estimation of total plasmin.

A solution of dried bovine trypsin inhibitor in buffer-saline was made of sufficient concentration to cause about 50 per cent inhibition of 0.0025 mg. of crystalline trypsin. Five serial twofold dilutions of this solution were made in buffer-saline and 1.0 cc. of each dilution was pipetted into a digestion tube. These tubes then received 2.0 cc. of buffer-saline and 1.0 cc. of a solution containing 0.00025 mg. of crystalline trypsin per cc. The tubes were incubated for

15 minutes to allow combination of trypsin and inhibitor. The residual active trypsin in each tube was then determined as previously described. Similarly 1.0 cc. of each dilution of inhibitor was added to a second series of digestion tubes containing 1.6 cc. of buffer-saline, 0.4 cc.

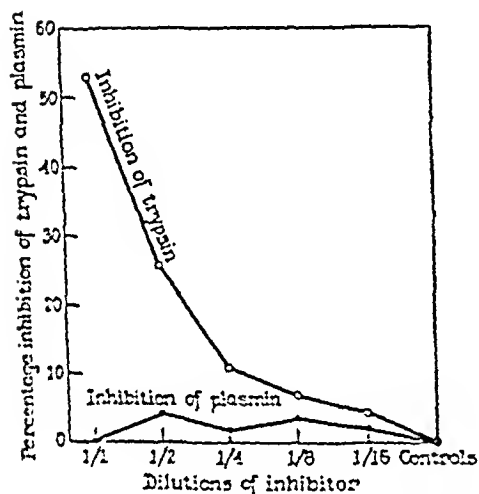


FIG. 3

FIG. 3. The effect of bovine trypsin inhibitor on the titration of total plasmin in normal human serum when the precautions described under Methods are used. Ordinates give the percentage inhibition of plasmin recorded thus ●—● and the percentage inhibition of trypsin recorded thus ○—○. Abscissae give dilutions of inhibitor and control titrations without any bovine inhibitor. Under these conditions the inhibitor did not significantly affect the titration of total plasmin.

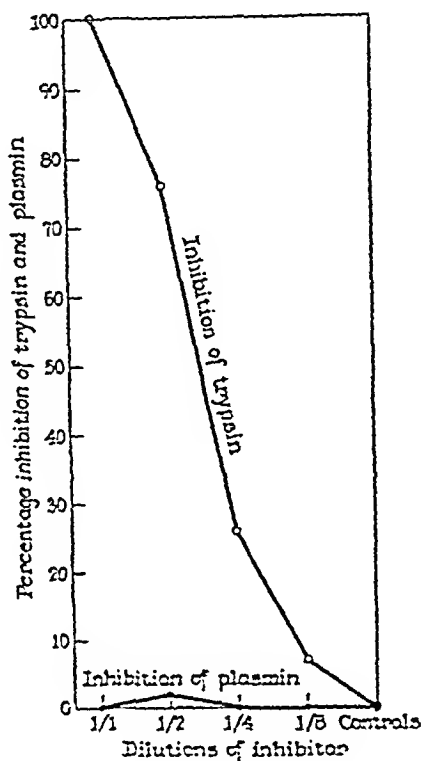


FIG. 4

FIG. 4. The effect of crystalline soya bean trypsin inhibitor on the titration of total plasmin by the method described. Ordinates and abscissae as in Fig. 3. A quantity of inhibitor which caused complete inhibition of trypsin did not affect the titration of total plasmin.

of normal human serum, and 1.0 cc. of streptokinase suitably diluted in buffer-saline solution. This operation was carried out in a cold water bath at about 4°C. and a stopper was added immediately in order to allow the smallest possible time for combination of plasmin and inhibitor. The tubes were then incubated for digestion as described under Methods. The whole experiment was repeated on the following day.

Fig. 3 gives the mean of the results of these titrations. It will be seen that, under the conditions described, undiluted inhibitor caused 53 per cent inhibi-

tion of trypsin but it failed to cause any inhibition of plasmin. The color released from azocoll by the two enzymes alone without any inhibitor averaged 36 units for trypsin and 40 units for plasmin.

A similar experiment was carried out using crystalline soya bean trypsin inhibitor instead of bovine inhibitor. In this experiment a quantity of inhibitor which was large enough to inhibit the trypsin completely did not cause any inhibition of plasmin. The color units released from azocoll by the two enzymes alone without any inhibitor were 63 units for trypsin and 52 units for plasmin. The result of this experiment is shown in Fig. 4.

These experiments add confirmation to the results already expressed in Fig. 2. The experiments recorded in Figs. 2, 3, and 4 all seem to show that the methods employed for the separate titration of total plasmin and of trypsin inhibitor give independent results and that the levels of plasmin determinations are not necessarily low because the serum contains a large amount of trypsin inhibitor.

The Effect of Spontaneously Activated Plasmin on the Titration of Trypsin Inhibitor

When titrating trypsin inhibitor in serum the dilution of 1/25,000 is sufficient to insure that autocatalytically activated plasminogen, which appears on storage of sera in the cold, will not supply sufficient plasmin to interfere with the test. This was demonstrated by an experiment in which twenty-three specimens of patients' sera, containing various quantities of total plasmin represented by color unit readings ranging from 32 to 56, were diluted 1/25,000 and tested for their action on 30 mg. of azocoll. Buffer-saline was used as the reference in this experiment. The light transmittance without any serum was 79 per cent; the mean transmittance for all the sera was 78.5 per cent with a variation of ± 2.0 or -1.5 . It is therefore evident that the quantity of serum used for titrations of trypsin inhibitor is sufficiently small to exclude any proteolytic action of plasmin.

The Effect of the Inhibitor in Streptokinase on the Estimation of Total Plasmin

Christensen and MacLeod (1945) have shown that concentrated solutions of streptokinase contain proteinase inhibitor. It is therefore necessary to titrate the streptokinase used for activation of plasminogen to determine the optimal concentration which will cause maximal activation of plasminogen without introducing enzyme inhibitors which are known to be present in concentrated preparations of streptokinase.

For this purpose serial twofold dilutions of a solution containing 25 mg. of partially purified streptokinase (S.K.A.I.) in 10.0 cc. of distilled water were pipetted into a series of digestion tubes in volumes of 0.4 cc. A volume of 0.4 cc. of normal human serum was added to each

tube; and the total volume was then made up to 4.0 cc. with buffer-saline solution at pH 7.8. After the addition of 30 mg. of azocoll and incubation for 30 minutes as previously described, the tests were read in the usual way. A similar experiment was set up in which patient's serum with an antistreptokinase titre of 1/2560 was used instead of normal human serum. The results of these experiments are given in Table II.

It will be seen from Table II, that, with normal human serum 0.25 and 0.125 mg. of this particular preparation of streptokinase produced maximal plasmin activity. Larger or smaller quantities were less effective. A quantity of 0.25

TABLE II

Determination of Optimal Quantity of Streptokinase for Use in Titration of Total Plasmin

Streptokinase mg.	Color units	
	Normal human serum	Patient's serum A.S.K. titre 1/2500
1.0	39	41
0.5	43	43
0.25	49	42
0.125	49	34
0.063	47	29
0.031	42	...
0.016	34	...
0.008	31	...
0.004	30	...

... = not done.

A.S.K. = antistreptokinase.

mg. of this streptokinase, therefore, allowed the largest possible margin of excess streptokinase for the titration of total plasmin in sera with low anti-streptokinase titres. With the patient's serum, however, maximal proteolytic activity was observed with a minimal quantity of 0.25 mg. of streptokinase. Smaller quantities of streptokinase gave rapidly diminishing proteolytic action which was presumably due to the large amount of antistreptokinase in the patient's serum. Since 1/2560 was, with a single exception, the highest anti-streptokinase titre encountered among the sera used in this investigation, a quantity of 0.25 mg. of streptokinase was selected for use in total plasmin titrations.

The Effect of Antistreptokinase on the Titration of Total Plasmin

The following experiment was carried out to determine whether or not 0.25 mg. of the partially purified streptokinase (S.K.A.I.) was sufficient to activate the plasminogen of patients' sera containing varying amounts of antistreptokinase.

Sera from twenty-two scarlet fever patients with antistreptokinase titres ranging from 0 to 1/5120 were titrated for total plasmin in the usual way. In addition, each of these sera was titrated for total plasmin when activated by half the usual quantity of streptokinase. The result of this experiment is given in Table III.

TABLE III

The Effect of Antistreptokinase on the Titration of Total Plasmin Using 22 Sera from Scarlet Fever Patients with Varying Quantities of Antistreptokinase in Their Blood

Antistreptokinase titres of sera (reciprocals of serum dilutions)	Proteolytic activity with 0.25 mg. of streptokinase (color units)	Proteolytic activity with 0.125 mg. of streptokinase (color units)	Increase or decrease of color units with half the usual quantity of streptokinase
0	46	46	0
0	53	54.5	+1.5
20	58.5	61	+2.5
20	40	47	+7.0
40	38	40.5	+2.5
40	52	47	-5.0
80	52	59.5	+7.5
80	56	62	+6.0
160	53	54	+1.0
160	46.5	46	-0.5
320	34	30	-4.0
320	39	44	+5.0
640	33	37	+4.0
640	39.5	40	+0.5
1280	43.5	42	-1.5
1280	31.5	29.5	-2.0
1280	41.5	36	-5.5
2560	35	31	-4.0
2560	34	38	+4.0
2560	43.5	39	-4.5
2560	43.5	40.5	-3.0
5120	33	29	-4.0

It will be seen from Table III that, with patients' sera which had A.S.K. titres ranging from 0 to 1/640, the usual quantity of 0.25 mg. of streptokinase was on the average slightly less efficient as an activator of plasminogen than half the usual quantity. Thus with A.S.K. titres of 1/640, or less, the average increase of proteolytic activity with half the usual quantity of streptokinase was represented by +2.0 color units. This was probably due to a slight excess of inhibitor in the usual quantity of streptokinase. On the other hand, with patients' sera which had A.S.K. titres higher than 1/640 the average loss of proteolytic activity with half the usual quantity of streptokinase was represented by -2.6 color units. This was probably due to the presence of sufficient antistreptokinase to cause partial neutralization of half the usual quantity of

activating streptokinase. It was therefore considered advisable to adopt 0.25 mg. of streptokinase as the standard quantity for activation of plasminogen in all titrations.

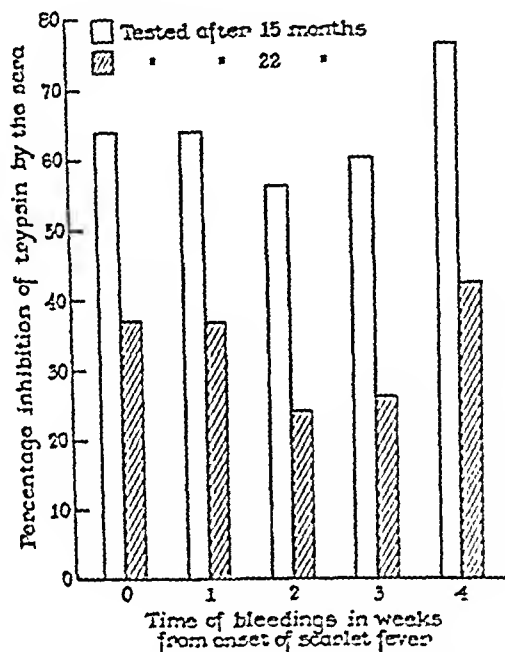


FIG. 5. Trypsin inhibitor in samples of serum taken at weekly intervals from a patient with scarlet fever. The sera, which were stored in the cold, were first titrated for trypsin inhibitor after 15 months and again after 22 months. Ordinates give percentage inhibition of trypsin. Abscissae give times when the patient was bled. The vertical columns give trypsin inhibitor levels after 15 and 22 months' storage.

The Lability of Trypsin Inhibitor in Serum

It is well known that trypsin inhibitor gradually disappears from samples of sterile serum which are stored in the cold. With animal sera this appears to be a relatively rapid process so that samples of serum which were originally capable of causing 80 per cent inhibition of trypsin may lose all their inhibitory power within a few months. On the other hand, the trypsin inhibitor of human serum appears to lose its inhibitory power much more slowly. Titrations of trypsin inhibitor in human sera, which will be described in a subsequent paper, showed that inhibitory action was still demonstrable after storage in the cold for periods ranging from 2 to 8 years. An example may be given of a patient who was bled in June, 1946. The trypsin inhibitor in this serum was first titrated after storage in the cold for 15 months when it caused 78 per cent inhibition of trypsin; after 22 months it caused 53 per cent inhibition and after 24 months,

46 per cent inhibition. A similar gradual loss of trypsin inhibitor in serum is illustrated in Fig. 5. In this case five samples of serum taken, at weekly intervals, from the same patient were titrated for trypsin inhibitor 15 months after bleeding and again 22 months after bleeding.

It will be seen from Fig. 5 that two approximately parallel curves were obtained with a loss of from 27 to 34 per cent of inhibitory power in all the sera during the 7 month interval. This is an unusually uniform fall in the inhibitory power of a series of samples of serum after storage in the cold. In experiments of this kind irregular changes in the inhibitory power of sera frequently occur so that two curves which are not of the same shape are obtained. This suggests that the trypsin inhibitor in fresh human serum may consist of different substances which are not equally stable when stored in the cold.

TABLE IV

The Stability of Total Plasmin in Sera Taken at Intervals from the Same Patient and Stored in the Cold

Total plasmin activity expressed as color units	
Stored 13 mos.	Stored 23 mos.
45	50
64	64
36	42
45	44
42	42
42	40

The Stability of Plasminogen in Serum

An attempt was made to determine whether a gradual loss of total plasmin occurs in human sera which are stored in the cold for long periods. A series of bleedings taken at intervals from a single patient were titrated for total plasmin after storage in the cold for 13 months. This titration was repeated 10 months later. The results of this experiment are given in Table IV.

It will be seen from Table IV that the largest variation between the two titrations was an apparent increase of 6 color units in one of the sera after storage in the cold for an additional period of 10 months. This variation represents a 5 per cent variation in light transmittance and it is within the limits of experimental error. It is therefore evident that plasminogen in serum is relatively stable when compared with trypsin inhibitor.

DISCUSSION

The object of these experiments was to devise methods by which separate determinations could be made of the total plasmin in human serum and of the

quantity of trypsin inhibitor in the same sample of serum. By this means it was hoped to be able to plot curves representing respectively the potential proteolytic activity of the serum and the inhibitory action of the same serum on proteolysis. When these curves are constructed in such a way as to follow the course of a patient's illness by means of weekly samples of serum, the relation of the two curves will indicate the periods of disease when potential proteolysis is at its maximum or at its minimum. Thus when the total plasmin level is high and the inhibitor level is low a high potential proteolytic activity may be assumed. Conversely a low total plasmin level coinciding with a high inhibitor level would indicate minimal potential proteolytic activity. Evidence has been presented in this paper that, by the methods described, an approximation to the separate titration of enzyme and inhibitor in the same serum can be made. The age of the serum is an important factor because plasminogen is relatively stable in comparison with trypsin inhibitor.

The titration of total plasmin in serum is carried out by activating the plasminogen with excess of streptokinase. This involves consideration of the plasmin inhibitor in streptokinase. Unconcentrated streptococcal filtrates cannot be used for activation because the necessary quantity of streptokinase would occupy too large a volume. It is therefore necessary to use concentrated and partially purified streptokinase in a solution containing the largest possible quantity of activator which will not cause inhibition of plasmin activity. It is also necessary to use a quantity of streptokinase which is large enough to combine with antistreptokinase in the patient's serum leaving sufficient excess of streptokinase for full activation of plasminogen.

The quantity of trypsin inhibitor in sera is estimated by allowing the inhibitor in 0.00016 cc. of serum to combine with 0.00025 mg. of crystalline trypsin and titrating the residual active trypsin. By using this small quantity of serum in trypsin inhibitor titrations the proteolytic action of the system is confined to residual active trypsin and proteolysis by plasmin is excluded.

SUMMARY

Methods are described for the separate titration of total plasmin and of trypsin inhibitor in human blood serum. Azocoll was used as the substrate in all experiments.

When titrating the total plasmin, interference by trypsin inhibitor was minimized by mixing together serum, streptokinase, and substrate in the cold before incubation was commenced.

When titrating the trypsin inhibitor, interference by plasmin was avoided by using small quantities of serum and of crystalline trypsin.

Experimental results show that with the methods employed neither the trypsin inhibitor in the serum nor the inhibitor in the streptokinase nor the

antistreptokinase in the serum significantly interfered with the results of titrations of total plasmin.

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QUANTITATIVE STUDIES ON THE TOTAL PLASMIN AND THE TRYPSIN INHIBITOR OF HUMAN BLOOD SERUM

II. VARIATIONS IN THE BLOOD CONCENTRATION OF TOTAL PLASMIN AND OF TRYPSIN INHIBITOR IN STREPTOCOCCAL DISEASES WITH SPECIAL REFERENCE TO RHEUMATIC FEVER

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Details of the methods employed to titrate separately the total plasmin and the trypsin inhibitor in human blood serum are given in the preceding paper (Todd, 1948); and the precautions necessary to allow independent titrations of total plasmin are described. Evidence is presented that by these methods an approximation to the separate titration of enzyme and inhibitor in a single sample of serum can be made.

The results of using these methods to follow variations in the levels of plasmin and inhibitor in blood taken at weekly intervals from scarlet fever patients are given in this paper. The same lots of azocoll, of streptokinase (S.K.A.I.), and of crystalline trypsin, described in the preceding paper, were again used in the following titrations. It was therefore possible, by employing these standardized reagents, to obtain comparable results for the different groups of streptococcal diseases investigated.

The source of the sera was described in the preceding paper. They were taken from young men of the same age group who were admitted to the hospital of the Great Lakes Naval Training Center with scarlet fever during March and April, 1946. All the bleedings were taken before breakfast in order to obtain clear sera and to avoid the high levels of inhibitor which are known to occur after meals. Samples of serum were taken on admission to the hospital and thereafter at weekly intervals from 380 scarlet fever patients. Only a small number of these sera could be titrated for plasmin and inhibitor owing to limited supplies of standardized azocoll and streptokinase. Sera from rheu-

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matic subjects with no evidence of active disease were also included in this investigation.¹

Four groups of cases were studied; the numbers of patients in each group were as follows: Uncomplicated scarlet fever, 7; scarlet fever followed by purulent complications, 6; scarlet fever followed by rheumatic fever, 15; rheumatic children with no evidence of active disease, 2.

The results of titrations of total plasmin and of trypsin inhibitor in the sera will be presented in a series of charts so that the relation of plasmin and inhibitor to each other can be compared in the different diseases investigated. In these charts the abscissae give the times when each patient was bled. Light lines indicate a final late sample of serum taken after the patient had returned to duty. The ordinates of the charts represent (1) the number of color units released by the activated plasmin of the serum from 30 mg. of azocoll, (2) the percentage inhibition of crystalline trypsin by the serum. The numerals obtained in the titration of plasmin and of inhibitor happen to fall in the same range. It is therefore convenient for purposes of comparison to plot curves of plasmin and inhibitor by reference to a single scale of ordinates. The erythrocyte sedimentation rate for each sample of blood is recorded in numerals at the top of each chart above the corresponding curves. These figures represent sedimentation after 1 hour determined by the Cutler method which, as is well known, gives lower readings than the Westergren method.

EXPERIMENTAL RESULTS

Titration of Sera from Patients with Uncomplicated Scarlet Fever

In Fig. 1 Charts 1 a to 1 g give the total plasmin and the trypsin inhibitor levels of sera from 7 patients with uncomplicated scarlet fever. Referring to individual cases it will be seen that 5 of the charts namely 1 a to 1 e inclusive show a marked tendency to parallelism between the two curves. In other words a rise or a fall in one of the curves is accompanied by a similar change in the other curve. It is also noteworthy that in all 5 cases the plasmin curves occupy higher levels on the charts than the corresponding inhibitor curves throughout the periods of observation. In Chart 1 f the inhibitor curve was higher than the plasmin curve during the first 2 weeks. This relationship was reversed by an increase of plasmin and a fall of inhibitor during the 2nd week. After 3 weeks the patient returned to duty; 14 weeks later, when the patient was finally examined, the relative levels of plasmin and inhibitor were similar to those found when he was first admitted to the hospital. In Chart 1 g a relatively high plasmin level was accompanied by a constant low inhibitor level during the first 3 weeks. During the 4th week a steep fall of plasmin level was accompanied by an increase of inhibitor.

¹ These sera were made available through the kindness of Dr. Sidney Rothbard.

Titration of Sera from Patients with Scarlet Fever and Purulent Complications

In Fig. 2 Charts 2 a to 2 f give the total plasmin and the trypsin inhibitor levels of sera from 6 scarlet fever patients who developed purulent complications. Chart 2 a gives plasmin and inhibitor levels of sera from a scarlet fever patient who developed a purulent streptococcal infection of the urinary tract.

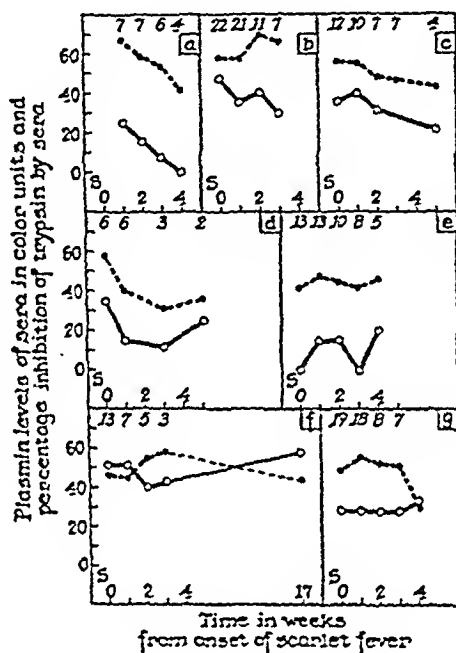


FIG. 1. Results of titrating sera from 7 patients with uncomplicated scarlet fever. Charts a to g. In all charts the ordinates give the plasmin levels of sera expressed as color units recorded thus ●—● and the percentage inhibition of trypsin by the sera recorded thus ○—○. Abscissae give time in weeks from the onset of scarlet fever which is recorded as zero. The numerals at the top of each chart, above the curves, give erythrocyte sedimentation rates (Cutler method) at the time of bleeding. S = uncomplicated scarlet fever.

The final sample of serum, taken after 3 weeks, had an antistreptokinase titre of 1/5000 which was the highest titre encountered among all the sera examined. In this case the curves of plasmin and inhibitor were roughly parallel; and the inhibitor levels were either slightly above or coincided with the plasmin levels. Charts 2 b to 2 e inclusive give plasmin and inhibitor levels of sera from 4 scarlet fever patients who developed otitis media. Chart 2 f gives plasmin and inhibitor levels of sera from a scarlet fever patient who contracted pharyngitis during the 3rd week due to hemolytic streptococci of a serological type different from the original infecting organism. He developed maxillary sinusitis 6 weeks after admission to the hospital.

Comparing Fig. 2 with Fig. 1, it will be seen that with purulent complications there is a tendency for the plasmin and inhibitor curves to be more closely approximated than in uncomplicated scarlet fever. Fig. 2 again shows a general tendency for the two curves to be roughly parallel although this is less

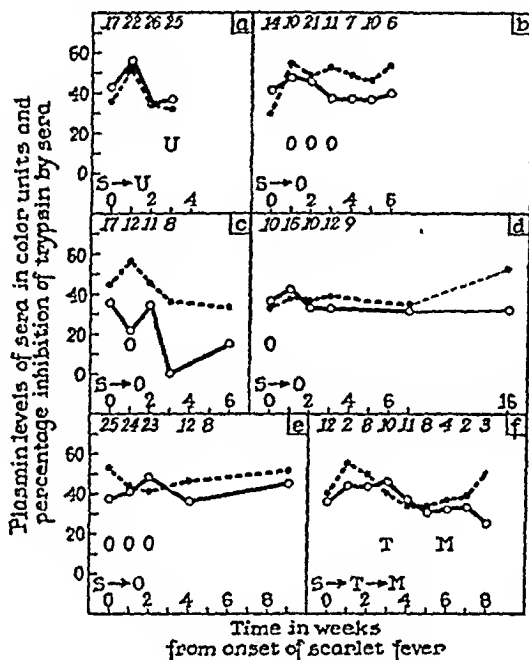


FIG. 2. Results of titrating sera from 6 patients with scarlet fever who developed purulent complications. Charts a to f. S → U = scarlet fever followed by purulent streptococcal infection of urinary tract. S → O = scarlet fever followed by otitis media. S → T → M = scarlet fever followed by tonsillitis due to reinfection by hemolytic streptococcus of different serological type, followed by maxillary sinusitis. O = week when otitis media was recorded. M = time when maxillary sinusitis was recorded.

evident than in uncomplicated scarlet fever. Among the purulent cases the plasmin curves are usually at a higher level than the inhibitor curves although this does not apply to the first 2 weeks in Charts 2 a and 2 d or to the 1st week in Chart 2 b. Charts 2 c and 2 f resemble those of rheumatic fever; they will be discussed in a subsequent section.

Titration of Sera from Patients with Scarlet Fever Followed by Rheumatic Fever

In Fig. 3 Charts 3 a to 3 f give the total plasmin and the trypsin inhibitor levels of sera from 6 patients with scarlet fever followed by rheumatic fever. The time of onset of the rheumatic attack is indicated by a vertical interrupted line. All the figures show a low plasmin level and a high inhibitor level at the

time of the rheumatic attack. In Charts 3 *a*, *b*, *c*, and *e* the maximal divergence between plasmin and inhibitor levels occurred at the time of the rheumatic onset. In Chart 3 *d* the plasmin level continued falling and the inhibitor level continued rising after the onset of rheumatic fever; the erythrocyte sedimentation rate also continued rising. In Chart 3 *f* there was a steep rise of the inhibitor level during the 6th week without any obvious reason.

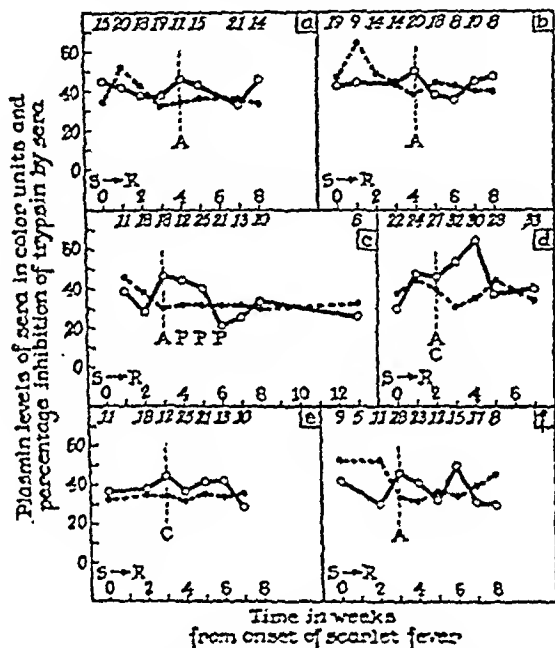


FIG. 3. Results of titrating sera from 6 patients with scarlet fever who developed rheumatic fever. Charts *a* to *f*. S → R = scarlet fever followed by rheumatic fever. The vertical interrupted lines indicate times of onset of rheumatic fever. A = week when arthritis appeared. C = week when carditis was recorded. P = week when pericarditis was recorded. In each case the rheumatic onset occurs when the plasmin level is low and the inhibitor level is high.

In Fig. 4 Charts 4 *a* to 4 *d* give the total plasmin and the trypsin inhibitor levels in sera from 4 patients with scarlet fever who developed rheumatic fever. In each case the rheumatic onset occurred when a relatively low plasmin level coincided with a relatively high inhibitor level. In Chart 4 *a* the plasmin level was at its lowest at the time of the rheumatic onset. At this time the inhibitor level was rising and it continued to rise until it reached its highest level at the time of the maximal erythrocyte sedimentation rate. In Chart 4 *b* the plasmin level fell slightly while the inhibitor level rose steeply until they almost coincided at the time of the rheumatic onset. In Chart 4 *c* the plasmin level fell

while the inhibitor level rose slightly until they coincided at the time of the rheumatic attack.

Chart 4 *d*, which is marked $S \rightarrow O \rightarrow R$, gives details of a particularly interesting case. This patient was admitted to the hospital with scarlet fever. During weeks 1 to 4 he suffered from otitis media; he was convalescent in the 5th week and then returned to duty. During the 9th week he was readmitted to the hospital with acute rheumatic fever. Group A type 17 hemolytic streptococci were isolated from his nasopharynx on two occasions during weeks

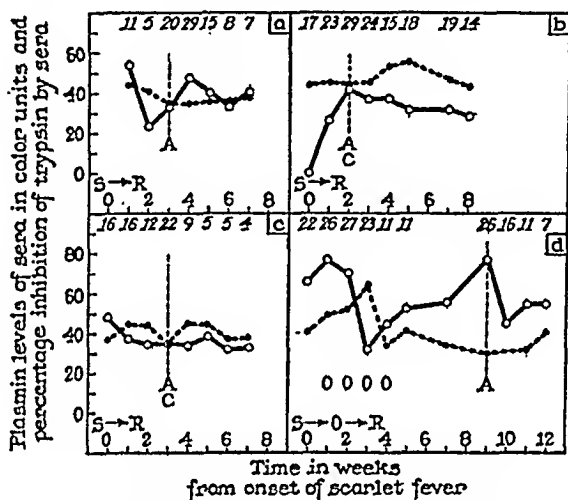


FIG. 4. Results of titrating sera from 4 patients with scarlet fever who developed rheumatic fever. Charts *a* to *d*. $S \rightarrow R$ = scarlet fever followed by rheumatic fever. $S \rightarrow O \rightarrow R$ = scarlet fever followed by otitis media followed by rheumatic fever. In each case the rheumatic onset occurs when a relatively low plasmin level coincides with a relatively high inhibitor level.

0 and 1; they were replaced during week seven by group A type 1 streptococci. On admission to the hospital he had an unusually large amount of inhibitor in his serum. During the course of otitis media, which lasted for 4 weeks, the plasmin level rose steeply and the inhibitor level fell steeply so that the curves crossed in the 3rd week. During convalescence from otitis media the plasmin level fell steadily while the inhibitor level rose steeply until they reached maximal divergence at the 9th week when rheumatic arthritis made its first appearance.

In Fig. 5 Charts 5 *a* to 5 *e* give the total plasmin and the trypsin inhibitor levels of sera from 5 patients with scarlet fever followed by rheumatic fever. Chart 5 *a* records uniformly low plasmin levels and high inhibitor levels throughout the whole course of the disease without any special change in the shapes of the curves at the time of the rheumatic attack. In Chart 5 *b* arthritis and carditis occurred at the end of the 2nd week when a high plasmin level coin-

cided with a low inhibitor level. This was followed by a fall of plasmin and a rise of inhibitor until their levels coincided at the time when the erythrocyte sedimentation rate was at its maximum. Thereafter, a fall of erythrocyte sedimentation rate was accompanied by a rise of plasmin and a gradual fall

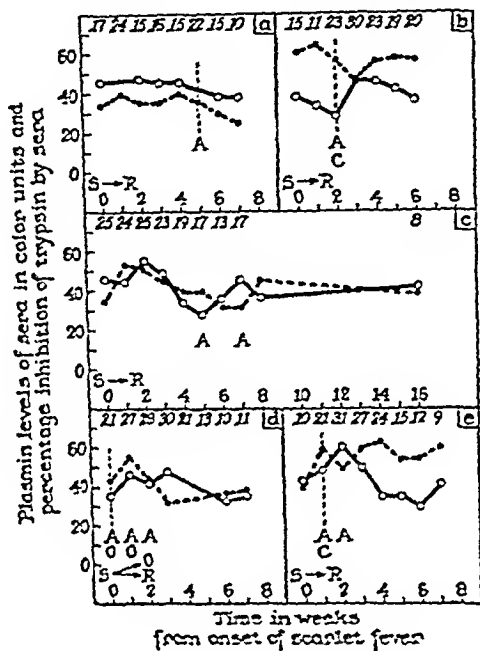


FIG. 5. Results of titrating sera from 5 patients with scarlet fever who developed rheumatic fever. Charts a to e. S → R = scarlet fever followed by rheumatic fever. S → O = scarlet fever followed by otitis media and rheumatic fever at the same time. A = arthritis. C = carditis. O = otitis media. In each case the inhibitor level was higher than the plasmin level, or the two levels coincided, at the time of the maximal blood sedimentation rate following the onset of rheumatic fever.

of inhibitor. In Chart 5c the vertical interrupted line is omitted owing to difficulty in determining the exact date of rheumatic onset. There was evidence of carditis during the first 2 weeks and the erythrocyte sedimentation rate was at its maximum during the 2nd week. At this time a high plasmin level coincided with a still higher inhibitor level. The first attack of arthritis occurred in the 5th week when the plasmin level had fallen slightly while the inhibitor level had fallen steeply, giving the unusual picture of arthritis occurring at a time when the inhibitor level was considerably below the plasmin level. A second attack of arthritis occurred a fortnight later; at this time the

plasmin and inhibitor levels were characteristically divergent with the lowest plasmin level recorded during the course of the disease.

Chart 5 *d*, which is marked S $\begin{matrix} \nearrow O \\ \searrow R \end{matrix}$, gives results for a patient who developed

otitis media and rheumatic polyarthrits at the same time during the first 2 weeks of scarlet fever. During this time the plasmin level was high while the

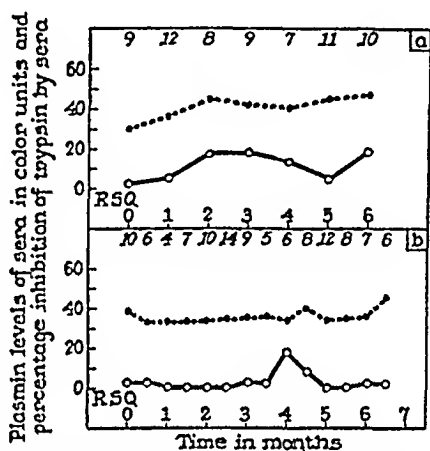


FIG. 6. Results of titrating sera from 2 rheumatic subjects with no evidence of rheumatic activity. Charts *a* and *b*. Abscissae give time in months during which children were under observation in the hospital; the date of admission is recorded as zero. The numerals at the top of each chart, above the curves, give erythrocyte sedimentation rates, Westergren method, at the time of bleeding. RSQ = rheumatic subject quiescent. In both cases the plasmin level was higher than the inhibitor level.

inhibitor level was relatively low. In the 3rd week the highest sedimentation rate was reached and at the same time the plasmin level was at its lowest and the inhibitor level at its highest.

In Chart 5 *e* rheumatic arthritis and carditis occurred during the 1st week with a high plasmin level and a relatively low inhibitor level. During the following week, when the sedimentation rate reached its maximum, the plasmin fell while the inhibitor rose to its highest point.

Titration of Sera from Rheumatic Subjects with No Evidence of Active Disease

In Fig. 6 Charts 6 *a* and 6 *b* give the total plasmin and the trypsin inhibitor levels of sera from 2 rheumatic children who did not show any signs of rheumatic activity or of infection during the 6 months while they were under observation and who had steadily falling antistreptolysin O titres during this

time. These sera were taken during the period October, 1940, to March, 1941; they had been stored in the cold for 8 years before titration for plasmin and inhibitor. The first of these children (Chart 6 *a*) was a girl aged 11 whose previous rheumatic attack occurred 3 years earlier. Samples of serum were taken about once a month during 6 months' observation. It will be seen from Chart 6 *a* that the plasmin levels were high throughout in relation to inhibitor levels. The second child was a boy aged 10 whose previous rheumatic attack occurred 17 months earlier. Samples of his serum were taken about twice a month. Chart 6 *b* shows plasmin levels which were again high in relation to inhibitor. The erythrocyte sedimentation rates for the two children were determined by the Westergren method.

Figs. 1 to 6 show that the charts of plasmin and inhibitor levels for this series of patients had a characteristic pattern in active rheumatic fever due to a fall of the plasmin level accompanied by a rise of the inhibitor level during the period of rheumatic activity. This rarely occurred in streptococcal diseases when rheumatic symptoms were not observed. Thus Fig. 1 shows that among 7 cases of uncomplicated scarlet fever, comprising 32 titrations of plasmin and inhibitor, the plasmin level was below the inhibitor level on only 4 occasions. In Fig. 2 which gives the plasmin and inhibitor levels of 6 scarlet fever patients with purulent complications there are 2 charts, namely 2 *c* and 2 *f*, which resemble those of rheumatic fever patients. Thus in Chart 2 *c* a rise of inhibitor coincided with a fall of plasmin in the 2nd week; in Chart 2 *f* the inhibitor curve rose slightly above the plasmin curve during weeks 3 and 4. Turning now to Figs. 3, 4, and 5 which give the plasmin and inhibitor levels for 15 patients with scarlet fever followed by rheumatic fever, it will be seen that in every case there was a change in the relative levels of the two curves coinciding either with the time of rheumatic onset or with the time of the maximal erythrocyte sedimentation rate after the onset of the rheumatic attack. This characteristic change was a fall of the plasmin level accompanied by a rise of the inhibitor level.

The possibility that the fall of the plasmin level at the onset of the rheumatic attack might be caused by a large amount of antistreptokinase in the blood at that time deserves consideration. It was shown in the preceding paper that the antistreptokinase titres of the sera did not influence the determination of plasmin levels under the experimental conditions adopted. Further evidence on this point is now supplied by the figures in Table I which give the antistreptokinase titres of the sera used in the plasmin and inhibitor titrations which are recorded in Figs. 1 to 5. Comparison of Table I with Charts 1 *a* to 5 *e* shows that there is no correlation between the antistreptokinase titres and the plasmin levels. In Table I, column 1 gives the case numbers used by Anderson, Kunitz, and McCarty (1948); column 2 gives the identification numbers of the

¹ These antistreptokinase titres were determined by Dr. H. C. Anderson and Dr. M. McCarty at the University of California, Los Angeles.

charts of plasmin and inhibitor levels recorded in this paper; the last five columns give the reciprocals of the antistreptokinase titres of the sera at weekly intervals following the onset of scarlet fever.

TABLE I

Reciprocals of the Antistreptokinase Titres of Sera Used for Plasmin and Inhibitor Titrations

Case No.	Chart	Titres at the following times in weeks dating from the onset of scarlet fever				
		0	1	2	3	4-6
279	1 a	80	80	160	320	320
49	1 b	40	40	40	80	...
304	1 c	40	80	80	80	80
108	1 d	0	40	...	160	160
123	1 e	40	...	80	160	160
131	1 f	20	320	320	320	...
288	1 g	40	40	40	40	80
22	2 a	20	40	640	5120	...
305	2 b	160	320	160	160	160
287	2 c	0	20	40	40	...
249	2 d	320	640	640	640	640
114	2 e	160	1280	1280	...	1280
196	2 f	40	80	80	1280	2560
16	3 a	20	20	160	320	320
61	3 b	320	320	2560	2560	2560
52	3 c	160	160	320	640	640
13	3 d	80	80	320	1280	2560
51	3 e	40	40	80	320	640
251	3 f	0	0	80	640	1280
120	4 a	80	80	320	640	1280
23	4 b	40	160	640	640	640
42	4 c	40	80	80	80	80
45	4 d	40	80	160	20	160
30	5 a	40	320	80	80	80
234	5 b	80	160	320	640	640
44	5 c	160	160	160	320	640
65	5 d	20	20	160	640	640
181	5 e	40	160	320	320	320

... = not done.

The characteristic patterns of the plasmin and inhibitor curves can be more easily recognized by referring to Fig. 7 which records on three charts the arithmetic means of the plasmin and inhibitor levels of the three different groups of scarlet fever patients. Thus Fig. 7 a gives the average levels for 7 cases of uncomplicated scarlet fever; Fig. 7 b gives averages for 6 cases complicated by suppuration and Fig. 7 c gives averages for 14 cases followed by rheumatic fever. The fifteenth case of rheumatic fever, namely 4d, was

omitted from Chart 7 *c* because the onset of rheumatic fever in this case occurred 10 weeks after admission to the hospital. Fig. 7 is constructed in a similar way to those already described. The ordinates give color units released by plasmin and the percentage inhibition of trypsin. The abscissae give weeks after admission to the hospital. The average time of rheumatic onset for the

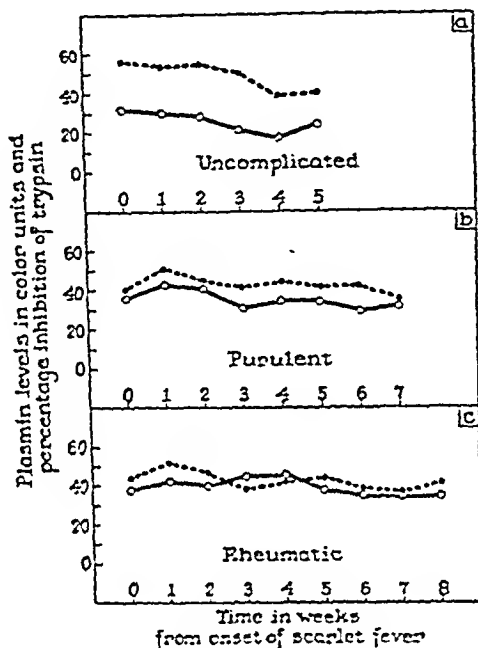


FIG. 7. Arithmetic means of plasmin and inhibitor levels of 7 patients with uncomplicated scarlet fever (Chart *a*); of 6 patients with scarlet fever followed by purulent complications (Chart *b*); and of 14 patients with scarlet fever followed by rheumatic fever (Chart *c*). In the uncomplicated and purulent groups the curves do not cross. In the rheumatic group the curves cross at the 3rd and 4th weeks.

14 cases represented in Chart 7 *c* was the 3rd week after admission to the hospital.

It will be seen from Chart 7 *a* that the average plasmin and inhibitor curves for uncomplicated cases of scarlet fever run approximately parallel courses with plasmin at a uniformly higher level than inhibitor. Chart 7 *b* shows that the average curves for patients with purulent complications are closer together than in Chart 7 *a* but they do not cross. Chart 7 *c* shows that the average curves for rheumatic fever cases cross at the 3rd week when plasmin is at its lowest and inhibitor is at its highest.

Fig. 7 allows a direct comparison of curves obtained with sera from the three different groups of patients based on the date of onset of scarlet fever but it

might tend to obscure the characteristic rheumatic pattern because the dates of onset of rheumatic symptoms after admission to the hospital varied from 1 to 6 weeks.

Fig. 8 was therefore constructed to give averages for plasmin and inhibitor levels in rheumatic fever cases based on the times of onset of rheumatic symptoms and on the dates of maximal erythrocyte sedimentation rates. Thus in Chart 8 *a* the vertical interrupted line gives the date of onset of rheumatic

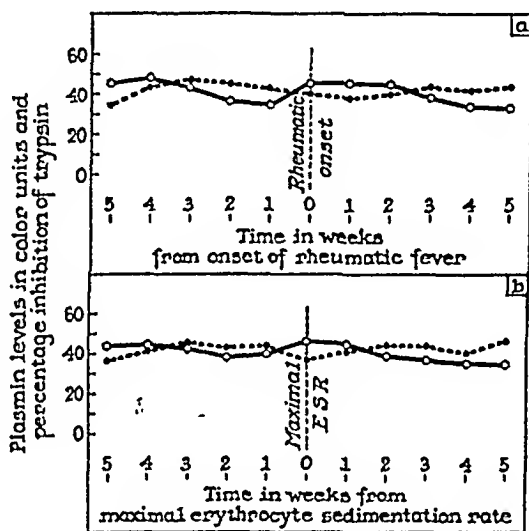


FIG. 8. Arithmetic means of the plasmin and inhibitor levels of 15 patients with scarlet fever followed by rheumatic fever. Abscissae in Chart *a* give time in weeks before and after the date of onset of rheumatic fever which is recorded as zero and indicated by a vertical interrupted line. In Chart *b*, which is based on the date of the maximal erythrocyte sedimentation rate after the onset of rheumatic fever, abscissae give time in weeks before and after the date of maximal erythrocyte sedimentation rate which is recorded as zero and indicated by a vertical interrupted line. ESR. = erythrocyte sedimentation rate.

symptoms. This point is recorded as zero on the base line and the preceding and succeeding weeks are numbered in succession dating from the time of the rheumatic attack. Similarly in Chart 8 *b* the vertical interrupted line gives the date of maximal blood sedimentation rates following the onset of rheumatic fever and it supplies the zero base line from which weekly intervals are numbered in both directions. Fig. 8 gives the averages of all of the 15 rheumatic cases examined.

It will be seen from Fig. 8 that the characteristic fall of plasmin and rise of inhibitor occur in both sets of curves. In Chart 8 *a* which is based on the first appearance of rheumatic symptoms, the inhibitor curve remains above the plasmin curve for 3 weeks; Chart 8 *b* which is based on maximal erythrocyte

sedimentation rates shows a preponderance of inhibitor over plasmin for 2 weeks.

An attempt was made to determine from the figures presented in Fig. 7 whether the divergence of plasmin and inhibitor curves which occurs in rheumatic fever is principally due to a fall of plasmin or to an increase of inhibitor.

Table II shows that when all the points on each of the three curves shown in Fig. 7 were averaged, uncomplicated scarlet fever had the highest plasmin and the lowest inhibitor levels. Comparing these figures for uncomplicated scarlet

TABLE II

Showing Quantitative Changes of Total Plasmin and of Trypsin Inhibitor in Uncomplicated Scarlet Fever, in Cases Complicated by Purulent Infections, and in Cases Followed by Rheumatic Fever

Chart	Cases included in each chart	Points on curves which were included in averages	Plasmin levels			Inhibitor levels			Differences of mean levels compared with those of uncomplicated scarlet fever	
			Minimum	Maximum	Mean	Minimum	Maximum	Mean	Plasmin	Inhibitor
7 a	Uncomplicated	Average of whole length of curve	39	57	49	18	31	26
7 b	Purulent	" " " " " "	37	51	42	30	43	36	-7	+10
7 c	Rheumatic	" " " " " "	38	52	42	35	44	39	-7	+13
7 a	Uncomplicated	At wk. 4 only	50	21
7 b	Purulent	" " " "	41	31	-9	+10
7 c	Rheumatic	" " " "	39	44	-11	+23

fever with the means of the corresponding plasmin and inhibitor levels shown in Charts 7 b and 7 c it will be seen that the purulent cases differed from uncomplicated cases in plasmin and in inhibitor respectively by -7 and +10 while the rheumatic cases differed by -7 and +13. There was therefore no very marked difference between purulent and rheumatic cases if the whole course of the disease was considered. On the other hand, a much greater difference appeared when the same procedure was applied to the averages of the 4th week only. Thus, when compared with uncomplicated scarlet fever, the purulent cases showed plasmin and inhibitor changes respectively of -9 and +10 and rheumatic cases -11 and +23. This shows that although the average level of plasmin is diminished and that of inhibitor increased during the whole course of both septic and rheumatic cases, yet this phenomenon is most conspicuous at the time of the rheumatic attack. It also shows that rise of inhibitor is greater than diminution of plasmin at the time when rheumatic manifestations appear.

DISCUSSION

Evidence was presented in the preceding paper (Todd, 1948) that the total plasmin in human serum could be estimated by determining its proteolytic action on azocoll after the plasminogen had been activated by a suitable excess of streptokinase. It was also shown that titrations of total plasmin in human serum were not materially affected either by the trypsin inhibitor in the serum if special methods were adopted or by the antistreptokinase in the serum if its titre was less than 1/5000.

The present paper gives the results of titrations of total plasmin and of trypsin inhibitor in sera taken at intervals from four different groups of patients, namely, uncomplicated scarlet fever, scarlet fever complicated by purulent infections, scarlet fever followed by rheumatic fever, and finally rheumatic children with no evidence of active rheumatic disease. The results of plotting curves of the total plasmin and of the trypsin inhibitor levels during the course of these diseases showed that in those cases where rheumatic symptoms were not manifested there was a general tendency for the plasmin and inhibitor curves to follow similar courses with corresponding elevations or depressions occurring at about the same time. This suggests the possibility that a rise or a fall in the level of total plasmin is normally compensated by a similar rise or fall of inhibitor so that excess of plasminogen is accompanied by excess of inhibitor while a low plasminogen content tends to coincide with a low inhibitor level. If such a compensatory mechanism exists, it appears to break down in rheumatic fever so that a fall of plasmin and a rise of inhibitor occur at the same time. In other words, the normal relative levels of plasmin and inhibitor are reversed in rheumatic fever and this change usually takes place either at the time when the first rheumatic symptoms appear or at a subsequent date when the blood sedimentation rate reaches its maximum.

The tentative hypothesis that plasmin and inhibitor are normally linked by a compensatory mechanism and that this mechanism breaks down in rheumatic fever cannot be substantiated until figures are available to show the relation of plasmin to inhibitor in sera taken at intervals from normal people and from patients with diseases other than those due to streptococcal infections. Unfortunately these titrations could not be included in the present investigation owing to limited supplies of standardized streptokinase and azocoll. The number of titrations of plasmin and inhibitor which were actually carried out was necessarily small for the same reason. It is hoped that a larger number of sera will be titrated later with fresh lots of streptokinase and azocoll.

Diseases caused by streptococci occupy a special category in an investigation of serum plasmin levels because the only substance which is known at the present time to be capable of activating plasminogen is the streptokinase elaborated by these organisms. This suggests the possibility that an increase of plasmin activity might occur in streptococcal diseases and particularly in

rheumatic fever since collagen is known to be affected in this disease. If this occurs, it should be demonstrable in plasmin titrations of rheumatic fever sera when azocoll, which contains a large amount of collagen, is used as the substrate. The present investigation seems to show that the opposite phenomenon actually occurs in rheumatic fever and that during the most acute phases of the disease the potential proteolytic activity is at its lowest. It is also noteworthy that rheumatic fever patients usually develop high titres of antistreptokinase in their blood which would tend to neutralize any streptokinase absorbed from the area of infection.

The parallel courses of total plasmin and inhibitor curves, which suggest that a compensatory mechanism may normally regulate the relative quantities of these substances in the blood, were most evident in uncomplicated scarlet fever. In these cases the plasmin curve was usually at a considerably higher level on the chart than the inhibitor curve. In suppurative complications of scarlet fever the two curves were relatively approximated due to an average lower plasmin level and an average higher inhibitor level. In cases that developed rheumatic fever there was, on the average, throughout the course of the disease, a plasmin level similar to that found in cases with suppuration and a slightly higher inhibitor level. In the 4th week, however, the cases with suppuration maintained their usual levels of plasmin and inhibitor in contrast to the rheumatic cases which showed a fall of plasmin accompanied by a marked increase of inhibitor which occurred only at this time when rheumatic symptoms most commonly occurred. This characteristic fall of plasmin and rise of inhibitor which occurred in rheumatic fever might be due to suspension or reversal of the compensatory mechanism which has already been tentatively suggested. Alternatively, it might be due to a qualitative change in the character of the inhibitor in rheumatic fever so that instead of being more active against trypsin than against plasmin it becomes more active against plasmin than against trypsin. This possibility raises the question of the character and stability of the trypsin inhibitor in serum. It is well known that fresh human serum contains a relatively large amount of trypsin inhibitor which gradually disappears when the serum is stored in the cold. The sera used in the present investigation had been stored in the cold for at least a year and the sera of the 2 quiescent rheumatic children for 8 years before they were titrated for plasmin and inhibitor. It was shown in the preceding paper (Todd, 1948) that no significant loss of total plasmin was observed in sera which had been stored in the cold for 11 months. On the other hand, a slow progressive loss of inhibitor was noted. It seems reasonable to suppose that an entirely different picture of the relations of total plasmin to trypsin inhibitor in the diseases investigated in this paper might have appeared if the charts had been prepared from data obtained by titrating fresh sera for plasmin and inhibitor. It is probable that the plasmin levels would have been similar to those recorded

and that the inhibitor levels would have been considerably higher than those recorded. Preliminary experiments with different reagents from those described in this paper confirm the observation that sera taken at weekly intervals from patients with uncomplicated scarlet fever and stored in the cold for long periods tend to give parallel curves of plasmin and of inhibitor levels. On the other hand, fresh sera of a similar nature tend to give plasmin and inhibitor levels which are reciprocally related. This difference between stored and fresh sera cannot be explained at present. It probably depends on the unknown nature of trypsin inhibitor in serum, on whether it is composed of a single substance or of two or more substances, and on differences in the relative stabilities of these possibly multiple substances.

It can, however, be stated that under the experimental conditions described, a marked fall of potential proteolytic activity occurred regularly at the time of rheumatic activity and that this phenomenon rarely occurred in streptococcal infections which showed no evidence of rheumatic disease.

No explanation can be offered at present for the low potential proteolytic activity of serum during the acute phase of rheumatic fever.

SUMMARY

The total plasmin and the trypsin inhibitor were titrated separately in samples of serum taken at weekly intervals from three different groups of scarlet fever patients: (a) those who did not develop any complications, (b) those who developed purulent complications, and (c) those who developed rheumatic fever.

When these determinations were plotted, it was found that the resulting curves showed characteristic patterns for each of the diseases investigated.

The uncomplicated cases had plasmin curves which were considerably higher on the charts than the inhibitor curves.

The septic cases had plasmin and inhibitor curves which were closer together on the charts.

The rheumatic cases had plasmin and inhibitor curves which were close together and which crossed at the time of rheumatic activity so that the inhibitor curve reached a higher level than the plasmin curve.

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THE SPECIFIC ANTIGENS OF VARIANTS OF SHIGELLA SONNEI

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PLATE 13

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The microorganism which has come to be known as *Shigella sonnei* was probably first isolated by Duval (1) in the United States. Some years later Kruse and his collaborators (2) and Baerthlein (3) independently isolated the same microorganism. It remained for Sonne (4), however, to demonstrate the etiological significance of this member of the dysentery group. The investigations of Sonne were confirmed and extended by Thjtta (5) and hnell (6). The early history of this microorganism has indeed been confused, as emphasized by Bojln (7) who states: "Certainly no other pathogenic microbe has been 'discovered' so many times as Sonne's bacillus."

Sh. sonnei was early recognized as readily undergoing variation. Baerthlein (3) as well as Sonne (4) and hnell (6) observed dimorphism of growth. However, Thjtta (5) was the first to point out the existence of two characteristic colony types, one of which did not agglutinate with the usual diagnostic antisera. Others (8-18) have also observed this characteristic variation and noted serological differences between the two colony types. Many investigators (11, 12, 15) have found that primary stool cultures are often mixtures of the two, and that the sera of patients suffering from Sonne dysentery infections frequently agglutinate both forms. Koser and Styron (14) were the first to apply the classical Smooth-Rough terminology to these variants of *Sh. sonnei*. The small, raised colony with regular edges described by Thjtta was termed the S variant, whereas the flat, irregular colony was termed the R variant.

Waller (17) has made the most extensive study of the dissociation of dysentery bacilli. He described four variants of *Sh. sonnei*. No. 1 was the typical S form; No. 2 grew in rough colonies but was antigenically identical with No. 1. The change from No. 1 to No. 2 appeared to be reversible. From No. 1 and No. 2 there could be obtained a third form, No. 3, which was antigenically different and which was believed to be an R variant. Still a fourth variant, No. 4, was described which was also rough but was antigenically feeble. Waller also noted the hair tuft colonies described by Thjtta (5) and questioned whether these colonies were in the process of dissociating from S to R.

Our understanding of the relationship among variants of *Sh. sonnei* has been considerably clarified by the recent work of Wheeler and Mickle (18). These investigators have emphasized that there are probably but three culturally and antigenically distinct types of *Sh. sonnei*. These they termed Phase I (smooth), Phase II, and

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Rough. In the present communication this terminology will be used, with some modification. It should be pointed out, however, that the term *phase* does not have the same significance as in the case of phase variation of the flagellar antigens of the *Salmonella* group.

Very little is known regarding the chemical nature of the antigens of *Sh. sonnei*. Haas (19) prepared a trichloroacetic acid extract of agar-grown bacilli and isolated a toxic substance found only in the smooth form. This substance was capable of eliciting antibodies in dogs which neutralized the toxin and agglutinated smooth bacilli. It gave negative biuret and sulfosalicylic acid, phloroglucinol and orcinol tests, but positive Molisch and ninhydrin tests. The material was not fully characterized.

In the present report the phase variation exhibited by *Sh. sonnei* will be scrutinized. It will be shown that, in the instance studied, variation did not involve a loss in ability of the microorganism to synthesize a somatic antigen, but was associated with a change in the immunological specificity of the latter.

EXPERIMENTAL

Methods

Cultivation of Bacteria.—The cultures used in this study were obtained from the United States Army Medical School and will be described later. The microorganisms were grown in a medium devised by Dole (20). Fifteen liters of the medium (containing 0.05 per cent glucose and 0.05 per cent phenol red) in a 5 gallon pyrex bottle was inoculated in the evening. The following morning 600 ml. of sterile 50 per cent glucose solution was added and the culture stirred mechanically. One ml. of tributyl phosphate was added to prevent foaming. Sterile air, dispersed by passage through a sintered glass disc, was bubbled through the culture at the rate of 500 ml. per minute. Five normal sodium hydroxide was added from a dropping funnel when necessary to maintain neutrality in the culture. Six to seven hours after the addition of glucose growth ceased, though acid production continued. Formalin was then added to a concentration of 1 per cent and the culture allowed to stand overnight at room temperature. The bacilli were then collected in a Sharples centrifuge and dried from the frozen state. The yield was usually about 35 gm. of dry microorganisms per 15 liters of culture.

The use of formaldehyde as a killing agent is open to criticism but it is known that treatment of other dysentery bacilli with this reagent does not alter the toxic or immunological properties of their somatic antigens.

Analytical Methods.—Nitrogen determinations were performed by the usual micro-Kjeldahl method. Phosphorus determinations were carried out according to the procedure of Allen (21). Reducing sugar was determined by the Shaffer-Somogyi method (22). Glucosamine was determined on acid hydrolysates by the method of Sørensen (23). Turbidimetric determinations of serologic activity of antigen preparations were made by means of the turbidimeter devised by Libby (24).

Serological Methods.—Antibacterial sera were prepared by injecting rabbits intravenously with graded doses of formalin-killed bacilli totaling 4.75 ml. of a 24 hour broth culture. Six injections in all were given at intervals of 3 to 4 days. The rabbits were bled on the 7th day after the last injection. Antisera to the somatic antigen preparations were obtained in a similar manner. A total of 850 micrograms was given.

The bacterial suspensions used in the agglutination tests were prepared by killing 18 hour broth cultures with 1 per cent formalin. The sedimented bacteria were washed twice and re-suspended in saline to the desired concentration. Occasionally preparations of the rough strain could not be used because of spontaneous agglutination, but most preparations were fairly stable after two saline washings.

The agglutination tests were performed by mixing equal volumes of the serum dilutions and the antigen suspension. The mixtures were incubated for 2 hours at 37°C. and allowed to stand overnight in the ice box. Readings were made the following morning.

Variants of Sh. sonnei

The Phase I culture as received from the United States Army Medical School proved to be a mixture of Phase I and II bacilli.

The Phase I colonies (Fig. 1) are round, raised, with entire edges and have a relatively smooth glistening surface. The colonies attain a diameter of 2 to 3 mm. after 18 to 24 hours incubation. Upon longer incubation many colonies develop irregular outgrowths,—the "hair tufts" of Thijssens (5). These latter consist of a mixture of Phase I and II bacilli. Growth of the Phase I microorganism in broth is uniformly turbid with relatively little tendency to settle. Agglutination with specific antiserum occurs as coarse clumps which do not break up upon shaking.

Under proper growth conditions, there may be obtained from the Phase I culture two variants which differ markedly in gross colony morphology but which are antigenically identical. The first of these, which we have termed Phase II_R, is identical with the Phase II variant of Wheeler and Mickle (18). When a Phase I culture is streaked on agar, most of the colonies are identical with the parent, but usually a few characteristic Phase II_R colonies are found. This same variant is observed on subculturing the "hair tuft" outgrowth from Phase I colonies. The Phase II_R colonies (Fig. 3) are flat, with an irregular outline and a rather granular although slightly glistening surface. The colonies attain a diameter of 5 to 6 mm. after 18 to 24 hours incubation at 37°C. They may become 1 to 2 cm. in diameter after longer incubation. Growth in broth is uniformly turbid but there will be some settling after 24 hours incubation. Agglutination with specific antiserum occurs as fine clumps which are easily dispersed upon shaking.

The second culture obtained from the Army Medical School proved to be Phase II, but because of its characteristically smooth colony morphology we have named it Phase II_S. Variants of this type probably develop spontaneously by mutation in Phase I cultures but because their colony morphology is so nearly like that of the Phase I variant they cannot be recognized. However, if a Phase I culture be grown in broth containing a small amount of chloroform, colonies of the Phase II_S variant will usually predominate when the culture is plated.

Phase II_S colonies (Fig. 2) are round, raised, with entire edges and a smooth glistening surface. The colonies are somewhat smaller than those of Phase I and attain a diameter of 1.5 to 2 mm. after 18 to 24 hours incubation at 37°C. Growth in broth is uniformly turbid with relatively little tendency to settle. Agglutination with specific antiserum occurs as fine clumps which are easily dispersed upon shaking.

The Rough variant was isolated by streaking on agar the unagglutinated bacilli in the supernatant of a Phase II_B culture which had previously been grown in the presence of homologous antiserum. The Rough colonies (Fig. 4) are similar to those of Phase II_R although the surface is somewhat more granular in appearance. Growth in broth occurs as fine clumps easily broken up upon shaking. There is a tendency for some preparations to show spontaneous agglutination in physiological salt solution.

TABLE I
The Quantitative Estimation of Phase Variation in Cultures of Shigella sonnei Phase I

Mutants found in a series of cultures				Mutants found in samples of a single culture			
Culture No.	Total No. of colonies counted	No. of mutant colonies counted	Per cent mutants	Sample No.	Total No. of bacteria counted	No. of mutants counted	Per cent mutants
1	509	8	1.6	1	541	4	0.74
2	531	10	1.9	2	545	3	0.55
3	536	5	0.93	3	550	4	0.73
4	500	13	2.6	4	539	5	0.92
5	505	7	1.4	5	515	4	0.77
6	540	5	0.93	6	513	2	0.39
7	501	71	14.0	7	534	7	1.3
8	541	4	0.74	8	531	6	1.1

Quantitative Aspects of Phase I to Phase II_R Variation

As pointed out previously, the Phase I culture used in these experiments was unstable; during growth in liquid or on solid media, Phase II_R variants were constantly being formed and possibly Phase II_B variants as well. No satisfactory methods have been developed for quantitative study of the phenomenon. In view of the presence of small amounts of Phase II antibody in all Phase I antisera it was thought desirable to obtain some information concerning the amount of Phase II contamination in Phase I cultures. Luria and Delbrück (25) have proposed the "fluctuation test" for detecting spontaneous mutation. According to this hypothesis, when mutations are rare, the frequency of mutation in each of a series of cultures should vary widely, while the number of mutants in several samples taken from the same culture should be identical within the counting error. This hypothesis is now widely accepted (26).

Observations of this type were made with the Phase I culture. Twenty-five tubes containing 5 ml. of neopeptone-beef heart infusion broth were inoculated with 0.1 ml. of a 10^8 dilution of an 18 hour broth culture inoculated from a single typical Phase I colony. This inoculum, according to a plate count, approximated one organism. After 18 hours incubation eight of these tubes showed growth. Appropriate dilutions of these cultures were prepared in sterile distilled water and 1 ml. distributed over the surface of five neopeptone agar plates (0.2 ml. per plate). The plates were incubated for 18 hours and the total number of colonies and of Phase II_R mutants were then counted. The results of this experiment are presented in Table I.

These and other data not tabulated indicated that almost all the Phase I cultures contained approximately 1 to 2 per cent of Phase II_R mutants. The greater variation in the number of mutants in samples taken from each of several cultures as compared with those removed from a single culture indicated the random distribution of mutation which occurs during growth of the micro-organism. Since the original inoculum was so small it is improbable that mutants were present when growth was initiated. In view of the high apparent

TABLE II

Agglutination Reactions of Variants of Shigella sonnei in Homologous and Heterologous Antisera

Antiserum prepared by immunization with:	Antigen	Final dilution of antiserum						
		1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400
Phase I bacilli	Phase I bacilli	++++	++++	++++	++++	+	0	0
	" II _S "	++	0	0	0	0	0	0
	" II _R "	+++	0	0	0	0	0	0
	R "	0	0	0	0	0	0	0
Phase II _S bacilli	" I "	0	0	0	0	0	0	0
	" II _S "	++++	++++	++++	++++	++++	0	0
	" II _R "	++++	++++	++++	++++	++++	0	0
	R "	+++	+++	+	0	0	0	0
Phase II _R bacilli	" I "	0	0	0	0	0	0	0
	" II _S "	++++	++++	++++	++++	+++	±	0
	" II _R "	++++	++++	++++	++++	+++	+	0
	R "	++++	++++	+++	+	0	0	0
Rough bacilli	" I "	0	0	0	0	0	0	0
	" II _S "	+++	+	0	0	0	0	0
	" II _R "	+++	0	0	0	0	0	0
	R "	++++	++++	++++	++++	++++	+++	+++

0 = no agglutination.

++++ = complete agglutination with clear supernatant.

mutation rate no attempt was made to apply the statistical computations developed by Luria and Delbrück (25).

Serological Properties of the Variants

The results of agglutination tests with the four variants in homologous and heterologous antisera are presented in Table II.

It will be noted that the Phase I variant agglutinated only in homologous antiserum. The Phase I antiserum used in these experiments was found to contain a small amount of antibody for the two Phase II but not for the Rough variant. It is probable that the presence of Phase II antibody in the Phase I antiserum was the result of the contamination of the Phase I vaccine with Phase II_R mutants, as shown above. This question will be discussed in greater detail later.

The agglutination tests also indicated that the Rough variant was to some extent contaminated with Phase II bacilli. Miller (27) has obtained evidence which substantiates this observation, for she has found that the R culture contained variants resistant to certain of the T series of bacteriophages. Some of these phage-resistant variants agglutinated only with Rough antiserum, whereas others agglutinated only with the Phase II antiserum. These data indicated that the Rough culture was a mixture rather than a single variant bearing a common antigenic component. Whether these variants were present because of the failure to purify the Rough culture by streaking or whether they

TABLE III
Toxicity in Mice of Variants of Shigella sonnei

Variant tested	Mg. bacteria injected		
	20	10	5
Phase I	DDD ₁₈	DDD ₁₈	DD ₁₈ S
Phase II _S	DDD ₁₈	DD ₁₈ S	DDD ₁₈
Phase II _R	DDD ₁₈	DD ₁₈ S	—
Rough	DDD ₁₈	D ₁₈ D ₁₈ S	D ₁₈ SS

D = death, the numerals representing the hours before death of the animal occurred.
S = survived. All animals observed for 3 days.

arose as a result of reverse mutation from Rough to Phase II is not yet established.

Toxic Properties of the Variants

Because of the difficulty in producing experimental dysentery in laboratory animals, there is but little information concerning the relative pathogenicity of the variants of *Sh. sonnei*. Some information concerning the relative toxicity of the variants for mice has, however, been obtained. These data are presented in Table III.

The results show that all the variants studied had approximately the same toxicity; a finding which differs from those of Hilgers (13) and Weil (28) who found the Phase I organism to be considerably more toxic than the other variants.

The Isolation and Characterization of the Specific Antigens

Isolation of the Phase I Antigen.—The use of 50 per cent aqueous pyridine for the extraction of the somatic antigens of the Flexner dysentery bacilli (29) was unsuccessful when applied to *Sh. sonnei*. Preliminary experiments showed that only traces of Phase I antigen were obtained when the corresponding microorganisms were extracted with this solvent. Good yields of the Phase I antigen

could be obtained, however, by extracting the bacilli with 50 per cent aqueous glycerol. Since preextraction of the microorganisms with 50 per cent pyridine removed considerable amounts of serologically inactive material, together with small amounts of a substance having Phase II activity, it was considered advisable to treat the Phase I organisms with aqueous pyridine prior to this extraction with 50 per cent glycerol.

128 gm. of dry bacilli were extracted twice for 24 hour periods and at 37°C. with 1200 and 800 ml. portions of 50 per cent aqueous pyridine. The bacilli were removed by centrifugation. The wet microorganisms were then extracted twice for 24 hours at 37°C. with 800 ml. portions of 50 per cent aqueous glycerol. After removal of the bacilli by centrifugation at 16,000 R.P.M., the combined glycerol extracts were filtered through a Berkefeld N filter. The filtrate was then dialyzed free of glycerol, concentrated *in vacuo*, and dried from the frozen state. 4.8 gm. of crude antigen was obtained. This material contained 5.11 per cent nitrogen and 3.25 per cent phosphorus.

4.7 gm. of the crude antigen was dissolved in 470 ml. of water and, after chilling, 0.5 volume of cold acetone was added with stirring. After standing overnight in the cold, the precipitate was removed. Acetone was added to the supernatant to give a concentration of 66 per cent. After standing 24 hours in the cold, the precipitate was collected by centrifugation, dissolved in 400 ml. of water, and 1 volume of cold acetone added. After 24 hours in the cold the small amount of precipitate which formed was removed by centrifugation, discarded, and to the supernatant cold acetone was again added to give a final concentration of 66 per cent. This precipitate, containing the active material, was collected by centrifugation, dialyzed, and dried from the frozen state. 1.95 gm. of substance was obtained which contained 6.2 per cent nitrogen, 3.9 per cent phosphorus, and some ribonucleic acid.

1.9 gm. of the above material was dissolved in 75 ml. of 0.01 M borate buffer at pH 7.8 and 4 mg. of crystalline ribonuclease added. The mixture was dialyzed against 2 liters of the 0.01 M borate buffer for 2 days at 37°C. in the presence of toluene. This process rendered most of the nucleic acid dialyzable. After a final dialysis against distilled water for 2 days the contents of the bag were concentrated to 75 cc. by pervaporation. Sodium acetate was then added to a concentration of 0.015 M, the pH adjusted to 7.0, and 1 volume of cold acetone added. After standing in the cold for 24 hours, a small amount of precipitate was removed by centrifugation, discarded, and cold acetone added to the supernatant to give a final concentration of 66 per cent. After 24 hours in the cold this precipitate was collected. The process of purification was repeated. The final precipitate of the Phase I antigen was dialyzed against distilled water, electro-dialyzed, and dried from the frozen state. 1.2 gm. of the product was isolated. Several lots of antigen were prepared in the above manner and all had essentially the same chemical and biological properties.

Isolation of the Phase II_s Antigen.—Preliminary experiments showed that some of the type-specific somatic antigen could be extracted from Phase II_s bacilli with 50 per cent aqueous pyridine, but the major part remained within the cell. Fifty per cent aqueous glycerol did not extract any of the antigen. However, it was found that a 7 M solution of urea would extract most of the antigen from bacilli which had previously been treated with aqueous pyridine.

10 gm. of dry Phase II_s bacilli were preextracted with aqueous pyridine as described above. The supernatant was then extracted in sufficient water so that after the addition of 804 ml. of urea the total volume reached 1200 ml. (7 M urea concentration). The extraction was

continued for 24 hours at 5°C. Higher temperatures did not extract greater amounts of antigen and resulted in the liberation of large amounts of ribonucleic acid. The microorganisms were removed from the urea solution by centrifugation at 16,000 R.P.M. for 30 minutes. The urea extract was filtered through a Berkefeld N filter, dialyzed free of urea, concentrated by pervaporation, and the solution dried from the frozen state. 1.57 gm. of crude antigen containing 6.0 per cent nitrogen, 3.3 per cent phosphorus, and considerable ribonucleic acid was obtained. The ribonucleic acid was eliminated by digestion and dialysis as previously described. From the dialysis sac 1.2 gm. of partially purified substance was obtained. The material contained 4.75 per cent nitrogen and 3.4 per cent phosphorus and a small amount of nucleic acid.

1.1 gm. of the partially purified antigen was dissolved in 110 ml. of 0.05 M sodium acetate and 0.5 volume of cold acetone added. After standing for 1 hour, the precipitate was removed by centrifugation at 15,000 R.P.M. for 30 minutes. This precipitated material, having very little serologic activity, was discarded. Sufficient cold acetone was added to the supernatant to give a concentration of 50 per cent. The precipitate was collected by centrifugation after

TABLE IV
Toxicity in Mice of the Specific Antigens of Phase I and II_s Shigella sonnei

Antigen tested	Micrograms of antigen injected				
	2000	1000	500	250	125
Phase I	DDD ₁₈	DD ₁₈ D ₂₄	D ₁₈ D ₂₄ S	D ₁₈ SS	SSS
Phase II _s	DDD ₁₈	DDD ₁₈	D ₁₈ SS	DD ₁₈ S	D ₁₈ SS

standing overnight in the cold. The material was dissolved in water and again precipitated at 50 per cent acetone concentration. The final precipitate was collected by centrifugation, dialyzed against distilled water, electro-dialyzed, and dried from the frozen state. 0.56 gm. of purified antigen was obtained. Various preparations have been made by the above procedure and all have essentially the same properties; these will be described below.

The Toxic and Serologic Properties of Phase I and II_s Antigens.—That the specific antigens of Phase I and II_s, *Sh. sonnei* had toxic properties comparable to those of the somatic antigens isolated from Flexner dysentery bacilli (30) is evident from the experiments recorded in Table IV. Inspection of the data presented in Table IV shows that the LD₅₀ for mice lay between 250 and 500 micrograms. The antigens of both variants were about equally toxic.

Antisera obtained from rabbits injected with the two purified antigens showed a high degree of specificity as can be seen from Table V. It will be noted, with one exception, that there was no evidence that the antisera obtained from rabbits injected with the purified Phase I and II antigens exhibited cross-reactions. The Phase II_s antiserum, at a dilution of 1:10, gave partial agglutination of the Rough variant, which may not, however, have been specific.

In Table VI are presented data on precipitin tests conducted with the Phase I and II_s antigens and the antisera of rabbits immunized with whole bacilli and with the two purified antigens. These tests show that in each instance the

purified antigens reacted specifically in the homologous immune sera, whether the serum was obtained from animals injected with whole bacilli, or with the highly purified lipocarbohydrate-protein complex. It is noteworthy that no cross-precipitin reactions were observed.

The Chemical and Physical Properties of Phase I and Phase II_s Antigens.—The antigens obtained from Phase I and II_s *Sh. sonnei* both gave positive biuret and Molisch tests. They were soluble in hydrochloric, acetic, and trichloroacetic acids, as are the somatic antigens of the other dysentery bacilli.

TABLE V

Agglutinins in Sera of Rabbits Immunized with Specific Antigens of Shigella sonnei Phase I and II_s

Antiserum prepared by immunization with:	Micro-organism tested:	Final dilution of antiserum									
		1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
		+++	+++	+++	+++	+++	+++	+++	++	0	0
Phase I antigen	Phase I	0	0	0	0	0	0	0	0	0	0
	Phase II _s	0	0	0	0	0	0	0	0	0	0
	Phase II _r	0	0	0	0	0	0	0	0	0	0
	Rough	0	0	0	0	0	0	0	0	0	0
Phase II _s antiserum	Phase I	0	0	0	0	0	0	0	0	0	0
	Phase II _s	+++	+++	+++	+++	+++	+++	+++	+++	+++	0
	Phase II _r	+++	+++	+++	+++	+++	+++	+++	+++	+++	0
	Rough	—	0	0	0	0	0	0	0	0	0

The gross analytical properties of the two antigens were not strikingly different as shown by the data presented in Table VII.

The Phase I and II_s antigens had similar nitrogen and phosphorus contents. The reducing sugar values, calculated as glucose, are maximum values obtained after 4 hours by hydrolysis with 2 N HCl at 100°C. Longer heating or higher acid concentrations resulted in considerably lower reducing sugar values. The analytical values presented for glucosamine were obtained by colorimetric analysis (23) after hydrolysis of the antigens for 18 hours at 100°C with 6 N HCl. In view of the low values for glucosamine, it is possible that this saccharide was not fully represented in the original substances and that the color developed was due to some other substance in the hydrolysis mixture. Actual isolation of glucosamine hydrochloride or a derivative is necessary in order to establish the presence of this sugar.

When the antigens were hydrolyzed with 0.1 N acetic acid for 4 hours at 100°C (31), they gave a mixture of insoluble protein and an acid-soluble carbohydrate fraction. In this respect, the antigens resemble the lipocarbohydrate-protein complexes obtained from other dysentery bacilli. However, it has not been sufficient material available for the determination of the type of carbohydrate present, and the physical and chemical properties were so characteristic that the antigens could be distinguished by rapid bioassay methods from the other antigens.

The ultraviolet absorption spectra of the two antigens were measured with the Beckman DU quartz spectrophotometer using solutions of 0.02 per cent in 0.5% trichloroacetic acid of a path length of 1 cm. The first characteristic peak

TABLE VI

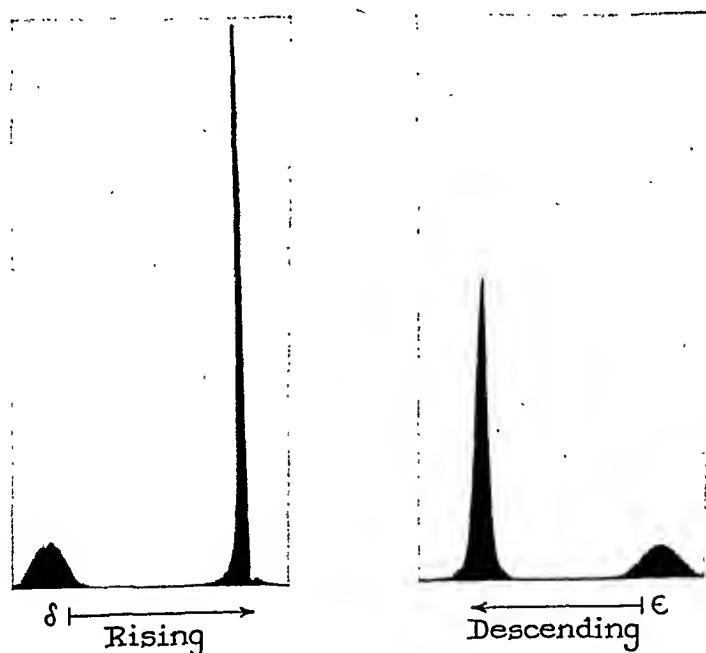
Precipitins in Sera of Rabbits Immunized with the Specific Antigens of Shigella sonnei Phase I and II_S

Antiserum prepared by immunization with:	Antigen tested	Final dilution of antigen			
		1:2000	1:10,000	1:50,000	1:250,000
Phase I antigen	Phase I	+++	++++	++++	++
	Phase II _S	0	0	0	0
Phase II _S antigen	Phase I	0	0	0	0
	Phase II _S	++++	++++	++	±

TABLE VII

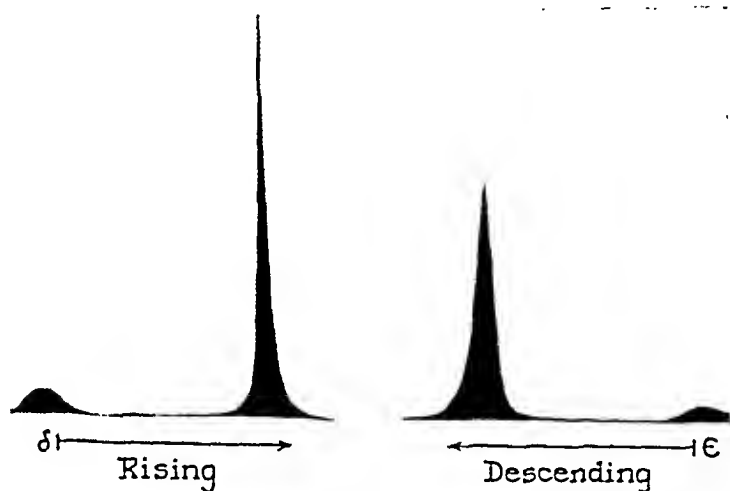
Analyses of the Specific Antigens of Phase I and II_S Shigella sonnei

Antigen	N	P	Reducing sugar after hydrolysis	Glucosamine
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Phase I	6.0	2.1	14.5	3.04
Phase II _S	5.45	3.1	23.4	4.19



TEXT-FIG. 1. Electrophoretic pattern of purified antigen obtained from *Shigella sonnei* Phase I.

ration showed a definite peak at a wave length of 260 millimicrons indicated that the substances were uncontaminated with nucleic acid. Electrophoretic analyses of the two antigens were made in the Tiselius apparatus using the Longworth scanning method. One per cent solutions of the antigens in veronal buffer at pH 8.6 and an ionic concentration of 0.1 were employed. The patterns obtained are shown in Text-figs. 1 and 2. It is evident from the accompanying figures that both antigens were homogeneous at this pH value. The mobilities were 3.2×10^{-6} cm.²/volt sec. for the Phase I antigen and 7.7



TEXT-FIG. 2. Electrophoretic pattern of purified antigen obtained from *Shigella sonnei* Phase II.

$\times 10^{-6}$ cm.²/volt sec. for the Phase II₂ antigen. While it would be desirable to make determinations at other values of pH, these data are considered sufficient to show the electrical properties of the two antigens.

DISCUSSION

That *S. sonnei* undergoes variation in a predictable manner is apparent from the data presented in this communication. The Smooth or Phase I micro-organism readily dissociated to yield two well defined variants which have been termed Phase II₁ and II₂. The latter were quite stable and were serologically distinct. They differed from one another in their colonial form and from the parent in respect to their immunological specificity. Prior to the re-

searches of Wheeler and Mickle (18), the Phase II_R variant was considered to be a Rough form of *Sh. sonnei*, but these investigators found that still another form could, under certain conditions, be obtained from their Phase II variant. Because of its cultural characteristics, the term Rough was assigned to this variant. The relatively rare occurrence of the Rough microorganism accounts for the failure of its earlier recognition. Although other colony forms of *Sh. sonnei* have been described (16, 17), they have not been encountered in this laboratory. In general, it would appear that the variants of *Sh. sonnei* most frequently encountered are those which have been termed Phases I, II (II_S and II_R), and Rough.

The data presented show that when *Sh. sonnei* undergoes variation the immunological change which occurs does not involve the loss of ability of the microorganism to elaborate a somatic antigen. The variant has acquired a new specificity yet it is still capable of carrying out the biochemical synthesis of a substance comparable in its gross chemical composition to that characteristic of the parent cell. In fact, the variation of *Sh. sonnei* may well involve only changes in the enzyme systems responsible for the synthesis of the somatic antigen. This change is reflected in a new and distinct serological specificity of the latter. It is probable that a detailed chemical study of the two antigens will reveal the nature of the differences responsible for the change in serological specificity. That these differences may well reside only in the chemical structure of the hapten components is not beyond the realm of possibility.

It will be observed from Table II that the antisera of rabbits injected with cultures of Phase I bacilli invariably showed some serological crossing with Phase II microorganisms. It is our opinion that this phenomenon occurred not because the Phase II antigen is an integral constituent of Phase I bacilli, but because the cultivation of the latter is accompanied by the production of small numbers of Phase II bacilli which arise through mutation. It has been clearly demonstrated that such mutations occur and hence the evidence is strong in support of this view. If, as it exists in the bacterial cell, the Phase I antigen contains a group which showed serological crossing in Phase II antisera, then the purified antigen should likewise show serological cross-reactions. This is not the case, however, for it has been demonstrated that the purified Phase I antigen is immunologically specific and gives rise to antibodies which show no serological crossing with Phase II antigen.

Phase variation has been shown to occur among bacilli of the dysentery group other than *Sh. sonnei*. Takita (32) studied a strain of Flexner Type V in detail, and found that two types of colonies could be isolated which were identical in gross morphology but were serologically distinct. One type, V_a, invariably bred true. The other, V_b, upon subsequent cultivation gave rise both to V_a and V_b variants. Serological tests showed that antisera prepared by injecting rabbits with cultures of the V_b variant contained antibodies which

agglutinated both V_a and V_b organisms. Antisera to the V_a variant, on the other hand, contained only homologous antibody. Boyd (33) and Weil, Farsetta, and Knaub (34) have confirmed and extended Takita's observations with other Flexner types.

All these investigators believed that the a variant represented a "degraded" Type V which had lost the b antigen, whereas the b variant contained both antigens. In view of the data presented in the present report, it is suggested that V_a represents a stable variant comparable to *Sh. sonnei* Phase II_s, and that V_b represents the smooth variant, cultures of which, due to mutation, contain both V_b and V_a variants. An antiserum prepared by immunization of animals with V_b bacilli would contain both antibodies, not because of the presence of a common antigen but because the V_b vaccine used would always contain some V_a bacilli arising through mutation.

Whether or not this phase variation of the Flexner bacilli is similar in nature to that of *Sh. sonnei* requires further investigation. The data presented here emphasize the need to establish the composition of vaccines used for the preparation of antisera. If a Smooth culture is undergoing mutation, a vaccine prepared from it will contain bacilli of the variant. The resulting antiserum, in addition to the type-specific antibody, will contain antibodies directed against the variant. The interpretation of data based upon the use of such antisera may lead to unwarranted assumptions.

SUMMARY

It has been shown that phase variation of *Sh. sonnei* is accompanied by changes in morphology and antigenic structure. Two mutants of the Phase I organism (II_s and II_n), which were studied, elaborate somatic antigens which are immunologically identical. The purified lipocarbohydrate-protein antigens of the Phase I and II_s microorganisms are chemically similar yet immunologically distinct and specific. By inference the same should hold true of the antigen of II_n, but it has not yet been investigated in this relation.

It is suggested that when *Sh. sonnei* undergoes variation from Phase I to II_s the immunological changes occurring are dependent upon a change in the enzyme systems responsible for the synthesis of the lipocarbohydrate-protein constituent.

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EXPLANATION OF PLATE 13

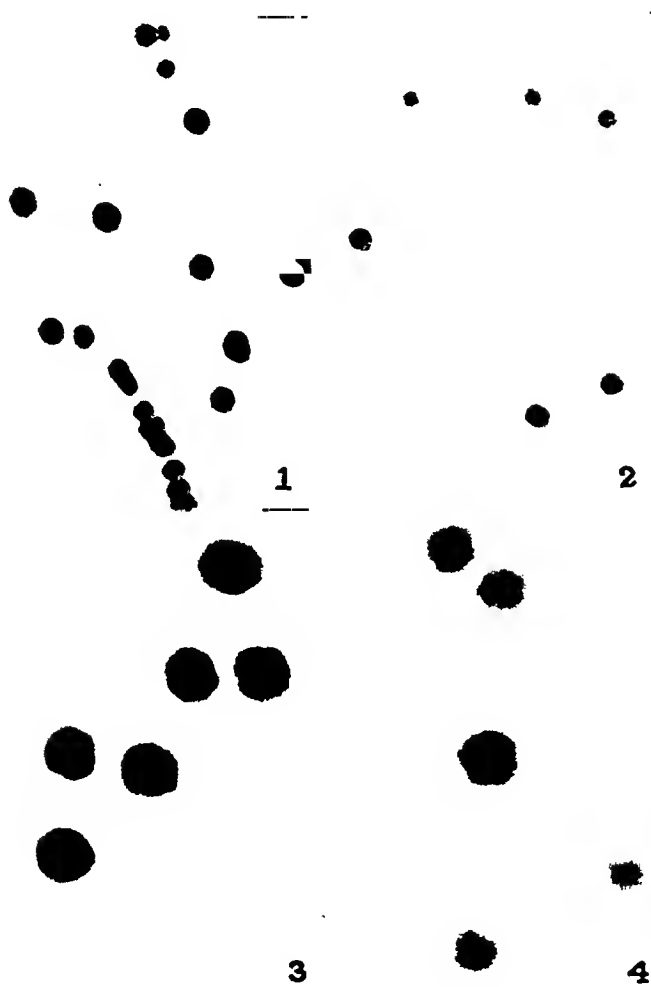
Colony morphology of the variants of *Shigella sonnei*. Cultures were grown for 24 hours on neopeptone-beef heart agar. $\times 3.25$.

FIG. 1. *Sh. sonnei*, Phase I—round, raised glistening colonies, 2 to 3 mm. in diameter.

FIG. 2. *Sh. sonnei*, Phase II_S—colony size slightly smaller than Phase I and more translucent.

FIG. 3. *Sh. sonnei*, Phase II_R—flat, irregular colonies with granular but slightly glistening surface, 5 to 6 mm. in diameter.

FIG. 4. *Sh. sonnei*, Rough—similar to Phase II_R but with more granular surface.



DIETARY EFFECTS ON ANEMIA PLUS HYPOPROTEINEMIA IN DOGS

I. SOME PROTEINS FURTHER THE PRODUCTION OF HEMOGLOBIN AND OTHERS PLASMA PROTEIN PRODUCTION*

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For several years this laboratory has contributed to the study of simultaneous anemia and hypoproteinemia (double depletion in dogs). The production of new hemoglobin and plasma protein in these animals can be controlled by the protein intake (1-3). The doubly depleted dogs (anemic and hypoproteinemic) give an opportunity to study the production response to various digests, pure food proteins, and amino acid mixtures. In the first years of this program it seemed reasonable to assume that some dietary factors would favor the production of hemoglobin over plasma protein or *vice versa*. There were hints that egg feeding did favor plasma protein production (3) but hemoglobin production was always in excess of plasma protein production, and the ratio of plasma protein to hemoglobin ran about 35 to 50 per cent.

Simple anemia produced by bleeding can be tolerated by the adult dog continuously during its entire life history and simple hypoproteinemia can be maintained for years but *double depletion* (anemia plus hypoproteinemia) is a severe strain on the dog and can be tolerated continuously only for a few months. One very important factor is appetite and distaste for the monotonous diet mixture terminates many experiments and impairs other. The basal diet contains the needed carbohydrate, fat, and dietary accessories. An excess of iron is added so that in these experiments the *only variable* is the type and amount of the specific food protein.

Our hope that in some measure we could control the blood protein output so that more plasma protein would be produced on one diet and more hemoglobin with another diet is now being realized. Some experiments with egg and egg albumin show even a little more plasma protein produced than hemoglobin. In contrast tests with meat and liver show three or four times as much hemoglobin produced as plasma protein (consult Summary Table 10). For example with meat feeding the output is large and hemoglobin is produced in great excess (Tables 6 and 7) but only limited bleeding can be done because of the

* We are indebted to E. I. Lilly and Company for aid in our feeding this work. We are indebted to Dr. James B. Allison, Dr. William H. Cole, and Dr. E. H. Barker of Rutgers University who initiated this cooperative investigation on evaluation protein foods.

low levels of plasma protein. The hemoglobin levels are twice as high as desired so we could readily introduce some whole plasma and remove much more hemoglobin and increase the stimulus for hemoglobin production—in other words the excess hemoglobin production would then be even greater (perhaps 5 to 1). Conversely with the egg diet which favors plasma protein production bleeding is limited by the low levels of hemoglobin (Tables 1 and 2). Here one could introduce washed cells, remove more plasma protein, and enhance the stimulus to plasma protein production—a modified plasmapheresis. In this way the ratio of plasma protein to hemoglobin might be raised to 150 to 200 per cent.

The whole response pattern is modified in some experiments by the labile exchange which can go on in a protein-depleted dog—for example a depleted dog can be maintained (4) for 3 months and more in protein balance on a sole protein intake of whole dog plasma. This means that plasma proteins must contribute to body proteins, including new hemoglobin. Some of these questions can be resolved by the use of radio carbon lysine given to label plasma proteins and these experiments are in progress or completed.

The albumin-globulin ratio is recorded under the experimental histories. As a general rule this ratio remains at 1.0 or above, in spite of the fact that depleted dogs tend to show a fall in the A/G ratio. In some of the experiments with egg albumin and with peanut flour the A/G ratio falls well below 1.0 to 0.7 or 0.6. Further study of the protein partition within the plasma will be reported subsequently.

Methods

The dogs used in these experiments are raised in the laboratory kennels and represent a white bull terrier and coach mixed strains. These animals are maintained under optimum dietary conditions and are kept under constant observation. They are protected from infections by proper housing, isolation, vaccination, and handling. Depletion of their blood proteins is produced as rapidly as is consistent with their well being. A non-protein diet plus frequent blood removal accomplishes depletion of both hemoglobin and plasma proteins within a 3 to 4 week period. During this depletion period these dogs lose considerable weight, making it desirable to produce the depletion in as short a time as possible. The desired "double depletion" represents a hemoglobin level of about 6.5 gm. per cent and a plasma level of approximately 4.5 gm. per cent. Below these levels the dogs' health and appetite suffer.

The basal non-protein diet consists of a biscuit containing adequate carbohydrates, fats, and minerals including an excess of iron and choline chloride. 100 gm. biscuit contains carbohydrate 73 gm., fat 13 gm., iron 78 mg., choline 70 gm. At times canned vegetables such as carrots or onions (low protein content) are added to maintain appetite during the depletion period. Vitamin additions to the diet consist of either a synthetic liquid vitamin mixture containing all known essentials (Lilly), or vitamin pills of like makeup or a dried yeast powder (Standard Brands Type 200 B) and a liver powder (Lilly) prepared from pig liver. The small amount of nitrogen these powders contain is added to the daily protein intake indicated in the tables.

Casein, peanut flour, wheat gluten, egg albumin, whole egg powder, and beef muscle powder (designated Rutgers University in the tables) represent protein materials prepared

for the "Bureau of Biological Research of Rutgers University for Comparative Studies on Methods of Evaluating Protein Foods." Description of these protein materials will appear elsewhere. These tests for potency concerning blood protein production are part of a cooperative investigation. Commercial casein and peanut flour are used for comparison experiments. Lactalbumin is a commercial product. Fresh beef muscle is ground lean round steak. Beef heart is boiled after fat is removed. Liver is fresh, cooked pig liver. Fresh egg albumin and yolk are separated and coagulated in a double boiler. Whole fresh egg is beaten thoroughly and coagulated in a double boiler. Canned salmon muscle is a commercial product, designated "pink Alaska salmon."

The experimental dogs are kept in metabolism cages in a separate room during the entire experimental period ranging from 3 to 7 weeks for each test as indicated in the tables. The dogs are weighed daily and their daily protein intake is accurately calculated. General technical procedures concerning the animals and experiments proper have been described elsewhere (2).

In the following tables for any given dog weekly periods run consecutively. Hemoglobin and plasma protein outputs are given in the tables in grams of material actually removed by bleeding and measured. Hemoglobin levels are those obtained by sampling 48 hours following blood removal in case of a single bleeding. In case of repeated bleedings during the week the hemoglobin level is obtained during the blood volume determination at the end of the week. Plasma protein levels represent the average of samples of each bleeding during the week. "Output per week" is the total hemoglobin or plasma protein removed during the week. *The figures in parentheses* are values indicating the "net corrected total output." These figures are the amounts of blood protein actually removed by bleeding, plus or minus the calculated amounts related to differences in the circulating levels of hemoglobin and plasma proteins as determined by blood volumes at the start and at the end of the given period.

Apparent discrepancy in figures in tables for protein intake and percentage food consumption is due to modified protein intake in diet mixtures as listed in the experimental histories of the dogs.

Experimental Histories (Tables 1 to 9, and 21 to 27 (Paper II))

The experimental history of the various dogs figuring in the tables in this and the succeeding paper is in the tables placed together for convenient reference. Dogs are numbered according to the year in which the experimental work was begun—for example, dog 40-32 started in 1940 and was the 32nd on the animal house list of that year. The experimental histories are arranged in chronological order.

Dog 40-32 (Tables 26, 22).—

Male Bull. Born August, 1940. Continuous anemia history Dec. 2, 1941, to May 26, 1942. Regular anemia experiments. Beginning weight 16.5 kilos, blood volume 1400 cc., plasma volume 493 cc. May 26, 1942—Daily diet of protein-free basal biscuit 350 gm., yeast 3 gm., liver extract powder 2 gm., reduced iron 400 mg. Blood protein depletion begun. Beginning blood volume 1200 cc., plasma volume 1030 cc., weight 16 kilos, plasma protein 6.2 gm. percent. Regular double depletion experiments with interspersed recovery periods. Aug. 26, 1942—Daily diet of protein-free basal biscuit 400 gm., canned onions 150 gm., yeast 3 gm., liver extract powder 2 gm. Beginning weight 22.9 kilos, blood volume 1776 cc., plasma volume 1318 cc., plasma protein level 6.3 gm. percent. Continued double depletion experi-

Nov. 8—*Wheat gluten* (Rutgers University) (Table 26). Daily diet of protein-free basal biscuit 600 gm., wheat gluten 40 gm., synthetic liquid vitamin mixture 8 cc. Nov. 16—Wheat gluten decreased to 30 gm. Nov. 22—Daily diet of basal biscuit 400 gm., wheat gluten 30 gm., dextrose 40 gm., synthetic liquid vitamin mixture 5 cc., onion juice 30 cc. Due to poor food consumption and weight loss the wheat gluten was incorporated into a biscuit and fed as such. Nov. 25—Wheat gluten biscuit 480 gm. equivalent to 40 gm. wheat gluten, synthetic vitamin pills 3. Plasma volumes 1116 cc., 1050 cc., 1080 cc., 1005 cc. A/G ratios 1.3, 1.2, 1.3, 1.2 (weekly intervals). Regular double depletion experiments continued with interspersed recovery periods. July 23, 1947—Daily diet of protein-free basal biscuit 350 gm., yeast 3 gm., liver extract powder 2 gm. Beginning weight 26.3 kilos, blood volume 2040 cc., plasma volume 1181 cc., plasma protein 6.7 gm. per cent. Blood protein depletion begun.

Sept. 19—*Casein* (commercial) (Table 22). Daily diet of protein-free basal biscuit 450 gm., casein 40 gm., lard 15 gm., synthetic liquid vitamin mixture 10 cc., dextrose 30 gm. Oct. 15—Reduced iron 600 mg. added to diet. Plasma volumes 1151 cc., 1146 cc., 1134 cc., 1159 cc., 1159 cc. A/G ratios 1.3, 1.4, 1.6, 1.5, 1.2 (weekly intervals).

Dog 40-34 (Table 9).—

Male bull terrier. Born August, 1940. Continuous anemia history Apr. 20, 1942, to Feb. 9, 1943: Regular anemia experiments. Beginning weight 14.3 kilos, blood volume 1084 cc., plasma volume 715 cc. Feb. 9, 1943—Daily diet of protein-free basal biscuit 450 gm., yeast 3 gm., liver extract powder 2 gm., reduced iron 400 mg. Blood volume 1206 cc., plasma volume 927 cc., weight 16.0 kilos. Blood protein depletion begun. Regular double depletion experiments with interspersed recovery periods.

Apr. 12, 1946—*Canned salmon muscle* (Table 9). Daily diet of protein-free biscuit 300 gm., yeast 3 gm., liver extract powder 2 gm., choline chloride 600 mg., canned salmon meat 170 gm. Apr. 25—Biscuit increased to 350 gm. Plasma volumes 940 cc., 912 cc., 927 cc., 854 cc. A/G ratios 1.0, 1.1, 1.3, 1.1 (weekly intervals). Dog in excellent condition.

Dog 40-36 (Tables 9, 4, 3).—

Male bull. Born August, 1940. Continuous anemia history Jan. 20, 1944, to Sept. 8, 1944. Regular anemia experiments. Daily diet of protein-free basal biscuit 500 gm., yeast 5 gm., reduced iron 600 mg. Beginning blood volume 1187 cc., plasma volume 943 cc., weight 14.9 kilos, plasma protein 5.8 gm. per cent. Blood protein depletion begun. Regular double depletion experiments with interspersed recovery periods.

Mar. 29, 1946—*Canned salmon muscle* (Table 9). Daily diet of protein-free basal biscuit 400 gm., salmon muscle 130 gm., yeast 3 gm., choline 600 gm. Apr. 12—Salmon increased to 160 gm., basal biscuit to 300 gm. Apr. 26—Salmon increased to 200 gm. May 10—Reduced iron 600 mg. added to diet. Plasma volumes 724 cc., 712 cc., 750 cc., 786 cc., 750 cc., 743 cc., 758 cc. A/G ratios 1.2, 1.2, 1.2, 1.1, 0.9, 1.0, 1.1 (weekly intervals). Continued double depletion experiments with interspersed recovery periods. Apr. 23—Daily diet of protein-free basal biscuit 400 gm., onions 100 gm., yeast 3 gm., liver extract powder 2 gm. Blood protein depletion begun. Plasma volume 823 cc., weight 19.8 kilos, plasma protein 6.2 gm. per cent.

May 16, 1947—*Egg albumin* (Rutgers University) (Table 4). Daily diet of protein-free basal biscuit 350 gm., egg albumin 35 gm., dextrose 30 gm., vitamin pills 3, lard 10 gm. June 13—Daily diet of protein-free basal biscuit 300 gm., egg albumin 30 gm., reduced iron 600 mg., dextrose 30 gm., lard 15 gm., biotin 0.4 mg., vitamin pills 3, choline 200 mg. Plasma volumes 844 cc., 818 cc., 774 cc., 882 cc., 792 cc. A/G ratios 1.0, 1.1, 1.2, 1.0, 0.7 (weekly intervals).

June 20—*Egg albumin* (fresh coagulated) (Table 3). Daily diet of protein-free basal biscuit 300 gm., egg albumin 200 gm., dextrose 30 gm., lard 10 gm., biotin 0.4 mg., yeast 3 gm., liver extract powder 2 gm., choline 200 mg., reduced iron 600 mg. June 27—Egg albumin increased

to 250 gm. July 1—Folic acid 5 mg. added to diet. Plasma volumes 782 cc., 778 cc., 729 cc., 763 cc. A/G ratios 0.8, 0.8, 0.8, 0.8 (weekly intervals).

Dog 41-52 (Table 6).—

Male bull adult born January, 1940. Maintained in laboratory kennel under optimum conditions. Aug. 8, 1945—Daily diet of protein-free basal biscuit 400 gm., salmon bread 75 gm., yeast 3 gm., liver extract powder 2 gm. Blood protein depletion begun. Beginning weight 18.4 kilos, blood volume 1579 cc., plasma volume 742 cc., plasma protein 6 gm. per cent. Aug. 30—Salmon bread omitted from diet. Continuous double depletion experiments. Mar. 3, 1947—Daily diet of protein-free basal biscuit 500 gm., yeast 3 gm., liver extract powder 2 gm. Blood protein depletion begun. Beginning blood volume 2249 cc., plasma volume 1090 cc., weight 26.2 kilos, plasma protein 5.9 gm. per cent.

May 2, 1947—*Beef muscle* (fresh, lean) (Table 6). Daily diet of protein-free basal biscuit 400 gm., beef muscle 200 gm., lard 15 gm., dextrose 20 gm., yeast 3 gm., liver extract powder 2 gm. May 29—Protein-free basal biscuit increased to 450 gm. June 6—Lard increased to 20 gm. Plasma volumes 1206 cc., 1084 cc., 1130 cc., 1120 cc., 1137 cc., 1138 cc. A/G ratios 1.0, 1.1, 1.2, 1.1, 1.2, 1.2 (weekly intervals).

Dog 41-53 (Table 25).—

Male coach. Born January, 1940. Continuous anemia history Feb. 16, 1943, to June 2. Regular anemia experiments. June 1, 1943—Regular double depletion experiments with interspersed recovery periods. Mar. 16, 1945—Daily diet of protein-free basal biscuit 500 gm., yeast 3 gm., liver extract powder 2 gm., reduced iron 600 mg. Beginning weight 27.5 kilos, blood volume 2540 cc., plasma volume 1222 cc., plasma protein 5.8 gm. per cent. Blood protein depletion begun.

Apr. 20—*Peanut flour* (commercial) (Table 25). Daily diet of protein-free basal biscuit 400 gm., peanut flour 60 gm., dextrose 50 gm., yeast 3 gm., liver extract powder 2 gm. Apr. 30—Protein-free basal biscuit increased to 450 gm. Plasma volumes 1098 cc., 966 cc., 1033 cc. A/G ratios 0.9, 0.9, 0.9 (weekly intervals). Continuous double depletion experiments.

June 1—*Peanut flour* (commercial, baked into biscuit) (Table 25). Daily diet of protein-free basal biscuit 150 gm., peanut flour biscuit 185 gm. equivalent to 60 gm. peanut flour, yeast 3 gm., liver extract powder 2 gm. Plasma volumes 897 cc., 908 cc., 820 cc. A/G ratios 0.8, 0.7, 0.6 (weekly intervals).

Dog 42-1 (Tables 26, 21, 6).—

Male bull adult. Born 1941. Maintained in laboratory kennels under optimum conditions. Continuous anemia history Feb. 14, 1945, to Aug. 8, 1945. Regular anemia experiments. Aug. 8—Daily diet of protein-free basal biscuit 450 gm., yeast 5 gm., liver extract powder 2 gm. Depletion of blood proteins begun. Beginning blood volume 1432 cc., plasma volume 964 cc., weight 19.1 kilos, plasma protein 6.1 gm. per cent. Regular double depletion experiments continued with an interspersed recovery period. Oct. 16, 1946—Daily diet of protein-free basal biscuit 500 gm., onions 150 gm., yeast 3 gm., liver extract powder 2 gm., reduced iron 600 mg. Depletion of blood proteins begun. Beginning blood volume 1947 cc., plasma volume 1148 cc., weight 23.8 kilos, plasma protein 7.2 gm. per cent.

Nov. 15—Wheat gluten (Rutgers University) (Table 26). Daily diet of protein-free basal biscuit 500 gm., wheat gluten 40 gm., synthetic liquid vitamin mixture 8 cc., dextrose 20 gm., reduced iron 600 mg. Nov. 12—Wheat gluten decreased to 35 gm., protein-free basal biscuit 400 gm. Nov. 25—Wheat gluten increased to 40 gm. Nov. 27—Due to poor food consumption the wheat gluten and wheat gluten was incorporated into a biscuit and fed as such. Wheat gluten biscuit replaced the 40 gm. wheat gluten. Plasma volumes 1148 cc., 1100 cc., 1044 cc.,

969 cc. A/G ratios 1.3, 1.1, 0.88, 0.9 (weekly intervals). Continued regular double depletion experiments.

Jan. 24, 1947—*Casein* (Rutgers University) (Table 21). Daily diet of protein-free basal biscuit 450 gm., casein 30 gm., dextrose 20 gm., synthetic vitamin pills 3, reduced iron 600 mg. Jan. 30—Choline 200 mg. added to diet. Feb. 11—Several skin lesions on front foot, given treatment. Synthetic liquid vitamin mixture 10 cc. added to diet. Feb. 14—Sugar mixture 30 gm. to replace dextrose 20 gm. Plasma volumes 902 cc., 889 cc., 878 cc., 872 cc. A/G ratios 0.94, 0.7, 0.7, 0.7 (weekly intervals). Continued regular double depletion experiments.

Mar. 7—*Beef muscle* (fresh lean uncooked) (Table 6). Daily diet of protein-free basal biscuit 350 gm., beef muscle 175 gm., synthetic liquid vitamin mixture 10 cc., dextrose 30 gm. Plasma volumes 906 cc., 863 cc., 925 cc., 945 cc. A/G ratios 0.74, 0.76, 0.78, 0.83 (weekly intervals).

Dog 44-16 (Tables 22, 8, 7, 6, 5).—

Female bull. Born October, 1944. Maintained in laboratory kennels under optimum conditions. July 23, 1947—Daily diet of protein-free basal biscuit 400 gm., yeast 3 gm., liver extract powder 2 gm. Blood protein depletion begun. Beginning weight 18.5 kilos, blood volume 1580 cc., plasma volume 804 cc., plasma protein 6.2 gm. per cent.

Aug. 15—*Casein* (commercial) (Table 22). Daily diet of protein-free basal biscuit 400 gm., synthetic liquid vitamin mixture 10 cc. Plasma volumes 743 cc., 702 cc., 665 cc., 715 cc., 704 cc. A/G ratios 1.0, 1.3, 1.6, 1.5, 1.4 (weekly intervals).

Sept. 19—*Beef heart* (fresh) (Table 8). Daily diet of protein-free basal biscuit 400 gm., cooked beef heart 118 gm., synthetic liquid vitamin mixture 10 cc., lard 10 gm. Oct. 14—Protein-free basal biscuit increased to 450 gm. Plasma volumes 680 cc., 732 cc., 735 cc., 752 cc., 759 cc. A/G ratios 1.5, 1.4, 1.3, 1.2, 1.3 (weekly intervals).

Oct. 24—*Beef muscle* (dried, powder) (Table 7) (Rutgers University). Daily diet of protein-free basal biscuit 450 gm., dried beef muscle powder 40 gm., synthetic liquid vitamin mixture 10 cc., lard 10 gm. Nov. 6—Protein-free basal biscuit increased to 500 gm. Nov. 15—Dextrose 40 gm. added to diet. Plasma volumes 690 cc., 702 cc., 694 cc., 714 cc., 690 cc. A/G ratios 1.2, 1.2, 1.5, 1.2, 1.3 (weekly intervals).—Continued regular double depletion experiments.

Dec. 22—*Beef muscle* (fresh) (Table 6). Daily diet of protein-free basal biscuit 450 gm., beef muscle 170 gm., synthetic liquid vitamin mixture 10 cc., lard 15 gm., dextrose 30 gm. Jan. 2—Protein-free basal biscuit decreased to 400 gm. Plasma volumes 622 cc., 676 cc., 650 cc., 654 cc., 639 cc. A/G ratios 1.3, 1.4, 1.3, 1.3, 1.3 (weekly intervals). Dog in good condition. Continuous double depletion experiments.

Apr. 23, 1948—*Egg yolk* (fresh, coagulated) (Table 5). Daily diet of protein-free basal biscuit 350 gm., egg yolk 210 gm., synthetic liquid vitamin mixture 10 cc. Apr. 30—Biotin 0.15 mg. added to diet. May 6—Protein-free basal biscuit increased to 400 gm., egg yolk to 220 gm. May 14—Reduced iron 600 mg. added to diet. Plasma volumes 640 cc., 641 cc., 620 cc., 707 cc. A/G ratios 1.5, 1.8, 1.4, 1.4 (weekly intervals).

Dog 45-2 (Tables 21, 23, 4, 2, 5).—

Male coach. Born March, 1945. Blood protein depletion begun. Nov. 2, 1946—Beginning weight 15.6 kilos, blood volume 1346 cc., plasma volume 666 cc. Plasma protein level 5.9 gm. per cent. Daily diet of protein-free basal biscuit 500 gm., yeast 3 gm., liver extract powder 2 gm. Continued double depletion experiment to Dec. 20. Plasma volume 784 cc. A/G ratio 1.7.

Dec. 20—*Casein* (Rutgers University) (Table 21). Daily diet of protein-free basal biscuit 400 gm., casein 35 gm., synthetic liquid vitamin mixture 8 cc., dextrose 20 gm. Dec. 28—

Casain increased to 40 gm. Plasma volumes 752 cc., 742 cc., 730 cc., 750 cc., 728 cc. A/G ratios 1.3, 1.4, 1.2, 1.3, 1.0 (weekly intervals).

Jan. 4, 1947—*Lactalbumin* (commercial) (Table 23). Daily diet of protein-free basal biscuit 450 gm., lactalbumin 47 gm., dextrose 20 gm., synthetic liquid vitamin mixture 8 cc. Feb. 14—Protein-free basal biscuit 400 gm., liquid vitamin mixture 10 cc., sugar mixture 30 gm. Plasma volumes 746 cc., 828 cc., 780 cc., 785 cc., 853 cc. A/G ratios 1.1, 1.6, 1.5, 1.2, 1.3 (weekly intervals). Continued regular double depletion experiments.

Apr. 5—*Egg albumin powder* (Rutgers University) (Table 4). Daily diet of protein-free basal biscuit 400 gm., egg albumin 35 gm., liquid vitamin mixture 10 cc., biotin 0.15 mg. Apr. 18—Egg albumin powder increased to 40 gm., protein-free basal biscuit 450 gm. May 2—Egg albumin powder 45 gm., biotin 0.22 mg., reduced iron 600 mg. Plasma volumes 821 cc., 722 cc., 703 cc., 780 cc., 682 cc. A/G ratios 1.0, 1.0, 1.2, 1.2, 1.0 (weekly intervals).

May 9—*Whole egg powder* (Rutgers University) (Table 2). Daily diet of protein-free basal biscuit 450 gm., whole egg powder 45 gm., biotin 0.22 mg., reduced iron 600 mg., synthetic liquid vitamin mixture 10 cc. May 29—Lard 15 gm. June 6—Lard omitted. Plasma volumes 668 cc., 762 cc., 732 cc., 718 cc., 786 cc. A/G ratios 1.0, 0.9, 1.0, 1.1, 1.0 (weekly intervals).

June 13—*Egg yolk* (fresh, coagulated) (Table 5). Daily diet of protein-free basal biscuit 400 gm., fresh egg yolk 200 gm., biotin 0.22 mg., reduced iron 600 mg., synthetic liquid vitamin mixture 10 cc., lard 15 gm. June 28—Fresh egg yolk increased to 250 gm. July 7—Fresh egg yolk 200 gm. Plasma volumes 727 cc., 776 cc., 779 cc., 834 cc., 808 cc. A/G ratios 1.3, 1.3, 1.3, 1.1, 1.3 (weekly intervals). Rest and recovery period. Dog normal.

Dog 45-3 (Tables 26, 27, 1).—

Male coach. Born March, 1945. Maintained in laboratory kennels under optimum conditions. Sept. 11, 1946—Daily diet of protein-free basal biscuit 400 gm., canned onions 150 gm., yeast 3 gm., liver extract powder 2 gm., canned salmon 100 gm. for 4 days. Blood protein depletion begun. Beginning plasma volume 657 cc., weight 15.1 kilos.

Oct. 5—*Wheat gluten* (Rutgers University) (Table 26). Daily diet of protein-free basal biscuit 450 gm., wheat gluten 30 gm., canned salmon 50 gm. (4 days). Oct. 7—Wheat gluten increased to 40 gm. daily. Plasma volumes 637 cc., 645 cc., 648 cc., 641 cc. A/G ratios 1.5, 1.4, 1.5, 1.5 (weekly intervals).

Nov. 22—*Cooked pig liver* (Table 27). Daily diet of protein-free basal biscuit 350 gm., cooked pig liver 110 gm., yeast 3 gm., liver extract powder 2 gm. Nov. 27—Pig liver increased to 125 gm. Dec. 3—Basal biscuit increased to 400 gm. Plasma volumes 569 cc., 582 cc., 563 cc. A/G ratios 1.3, 1.4, 1.3 (weekly intervals).

Dec. 13—*Whole coagulated egg* (Table 1). Daily diet of protein-free basal biscuit 350 gm., coagulated whole egg 200 gm., yeast 3 gm. Dec. 29—Basal biscuit increased to 400 gm., yeast replaced by synthetic vitamin pills 3. Dec. 27—Basal biscuit reduced to 300 gm., biotin 0.2 mg. added to daily diet. Jan. 3, 1947—Beginning skin lesions. Plasma volumes 590 cc., 592 cc., 676 cc. A/G ratios 1.4, 1.1, 1.1 (weekly intervals).

Dog 45-6 (Tables 1, 21, 23, 2, 4).—

Female coach. Born March, 1945. Maintained in laboratory kennels under optimum conditions. Sept. 11, 1946—Daily diet of protein-free basal biscuit 450 gm., canned onions 150 gm., yeast 3 gm., liver extract powder 2 gm. Blood protein depletion begun. Beginning weight 14.91 lb. Plasma volume 1380 cc., plasma volume 652 cc. Plasma protein 5.0 gm. per cent. Regular double depletion experiments.

Nov. 6, 1946—*Whole coagulated egg* (Table 1). Daily diet of protein-free basal biscuit 425 gm., whole coagulated egg 150 gm., synthetic vitamin pills 3. Dec. 14—*Whole coagulated*

egg increased to 180 gm., biotin 0.2 mg. Plasma volumes 633 cc., 620 cc., 648 cc., 662 cc. A/G ratios 1.4, 1.2, 1.2, 1.6 (weekly intervals).

Jan. 3, 1947—*Casein* (Rutgers University) (Table 21). Daily diet of protein-free basal biscuit 400 gm., casein 35 gm., synthetic vitamin pills 4. Plasma volumes 585 cc., 581 cc., 586 cc., 578 cc., 621 cc. A/G ratios 1.4, 1.2, 1.1, 1.1, 1.4 (weekly intervals). Continued regular double depletion experiments.

Feb. 7, 1947—*Lactalbumin* (commercial) (Table 23). Daily diet of protein-free basal biscuit 450 gm., lactalbumin 40 gm., sugar mixture 40 gm., choline 200 mg., synthetic vitamin pills 3. Feb. 14—Sugar mixture decreased to 30 gm. Feb. 20—Sugar mixture replaced by dextrose 30 gm., choline omitted. Plasma volumes 606 cc., 598 cc., 603 cc., 600 cc., 598 cc. A/G ratios 1.8, 1.7, 1.8, 1.5, 1.2 (weekly intervals).

Mar. 14, 1947—*Whole egg powder* (Rutgers University) (Table 2). Daily diet of protein-free basal biscuit 375 gm., whole egg powder 40 gm., dextrose 30 gm., synthetic vitamin pills 3, choline 200 mg. Mar. 28—Biotin 0.15 mg. daily added to diet. Plasma volumes 582 cc., 626 cc., 606 cc., 612 cc., 582 cc. A/G ratios 1.5, 1.6, 1.4, 1.7, 1.7 (weekly intervals).

Apr. 18, 1947—*Egg albumin* (Rutgers University) (Table 4). Daily diet of protein-free basal biscuit 325 gm., egg albumin powder 40 gm., biotin 0.15 mg., synthetic vitamin pills 3, choline 200 mg. Apr. 26—Biscuit increased to 375 gm. and reduced iron 600 mg. daily added to diet. Plasma volumes 589 cc., 554 cc. A/G ratios 1.6, 1.5 (weekly intervals). Dog in good condition throughout these experiments.

Dog 46-4 (Table 23).—

Male bull. Born December, 1946. Maintained in laboratory kennels under optimum conditions. Sept. 10, 1947—Daily diet of protein-free basal biscuit 450 gm., yeast 3 gm., liver extract powder 2 gm. Blood protein depletion begun. Beginning weight 16.5 kilos, blood volume 1255 cc., plasma volume 710 cc., plasma protein 5.5 gm. per cent.

Nov. 7—*Lactalbumin* (commercial) (Table 23). Daily diet of protein-free basal biscuit 450 gm., lactalbumin 48 gm., dextrose 50 gm., lard 10 gm., synthetic liquid vitamin mixture 10 cc. Nov. 17—Basal biscuit decreased to 400 gm. Nov. 29—Basal biscuit decreased to 350 gm., lactalbumin to 35 gm. Dec. 5—Lactalbumin increased to 40 gm. Reduced iron 600 mg. added to diet. Plasma volumes 778 cc., 762 cc., 724 cc., 690 cc., 686 cc. A/G ratios 1.7, 1.6, 1.3, 1.2, 1.3 (weekly intervals).

Dog 46-5 (Tables 24, 8, 9).—

Male bull. Born December, 1946—Maintained in laboratory kennels under optimum conditions. Sept. 10, 1947—Daily diet of protein-free basal biscuit 450 gm., yeast 3 gm., liver extract powder 2 gm. Blood protein depletion begun. Beginning weight 14.3 kilos, blood volume 1060 cc., plasma volume 640 cc., plasma protein 5.5 gm. per cent.

Oct. 3—*Peanut flour* (Rutgers University) (Table 24). Daily diet of protein-free basal biscuit 500 gm., peanut flour 40 gm., synthetic liquid vitamin mixture 10 cc. Oct. 6—Liquid vitamin mixture replaced by synthetic vitamin pills 3. Oct. 17—Basal biscuit decreased to 400 gm., peanut flour to 30 gm. Plasma volumes 574 cc., 518 cc., 480 cc. A/G ratios 1.3, 0.9, 1.1 (weekly intervals).

Oct. 24—*Beef heart* (fresh) (Table 8). Daily diet of protein-free basal biscuit 450 gm., cooked beef heart 124 gm., synthetic liquid vitamin mixture 10 cc., lard 10 gm. Oct. 31—Basal biscuit increased to 500 gm. Plasma volumes 575 cc., 656 cc., 598 cc., 569 cc., 584 cc. A/G ratios 1.0, 1.2, 1.5, 1.4, 1.2 (weekly intervals).

Nov. 28—*Salmon muscle* (canned) (Table 9). Daily diet of protein-free basal biscuit 400 gm., salmon muscle 125 gm., synthetic liquid vitamin mixture 10 cc., dextrose 40 gm., lard 10 gm. Dec. 12—Basal biscuit 350 gm. Dec. 19—Synthetic liquid vitamin mixture replaced by synthetic vitamin pills 3, basal biscuit 350 gm. Dec. 26—Salmon muscle 150 gm., lard 15 gm. (3 days). Plasma volumes 636 cc., 646 cc., 620 cc., 608 cc., 606 cc. A/G ratios 1.4, 1.3, 1.1, 1.1, 1.2 (weekly intervals).

Dog 46-9 (Tables 27, 3).—

Male coach born September, 1946. Maintained in laboratory kennels under optimum conditions throughout growth period. Nov. 5, 1947—Daily diet of protein-free biscuit 500 gm., yeast 3 gm., liver extract powder 2 gm., lard 10 gm., canned onions 100 gm. Blood protein depletion begun. Beginning weight 18.7 kilos, blood volume 1580 cc., plasma volume 761 cc., plasma protein 5.0 gm. per cent.

Dec. 5—*Pig liver* (cooked) (Table 27). Daily diet of protein-free basal biscuit 550 gm., lard 15 gm., dextrose 40 gm., pig liver 110 gm., synthetic liquid vitamin mixture 10 cc. Beginning plasma volume 844 cc. Dec. 24—Pig liver 105 gm. Plasma volumes 826 cc., 872 cc., 841 cc., 835 cc., 865 cc. A/G ratios 1.3, 1.3, 1.4, 1.1, 1.5 (weekly intervals). Continuous double depletion experiments.

Apr. 23, 1948—*Egg albumin* (fresh, coagulated) (Table 3). Daily diet of protein-free basal biscuit 350 gm., egg albumin 220 gm., synthetic liquid vitamin mixture 10 cc., biotin 0.15 mg. Apr. 28—Egg albumin increased to 240 gm., protein-free basal biscuit to 400 gm. May 6—Egg albumin increased to 250 gm. May 14—Egg albumin increased to 260 gm. Plasma volumes 805 cc., 902 cc., 887 cc., 844 cc. A/G ratios 1.0, 1.3, 1.3, 1.4 (weekly intervals).

Dog 46-10 (Table 7).—

Male coach. Born September, 1946. Maintained in laboratory kennels under optimum conditions throughout growth period. Sept. 24, 1947—Daily diet of protein-free basal biscuit 500 gm., yeast 3 gm., liver extract powder 2 gm. Blood protein depletion begun. Beginning weight 15.1 kilos, blood volume 1163 cc., plasma volume 620 cc., plasma protein 5.9 gm. per cent.

Oct. 17—*Dried beef muscle* (Rutgers University) (Table 7). Daily diet of protein-free basal biscuit 400 gm., dried beef muscle 40 gm., synthetic liquid vitamin mixture 10 cc. Oct. 24—Basal biscuit 450 gm. Nov. 7—Basal biscuit 500 gm. Plasma volumes 583 cc., 648 cc., 638 cc., 658 cc., 650 cc. A/G ratios 1.1, 1.1, 1.3, 1.4, 1.3 (weekly intervals).

Dog 46-22 (Table 27).—

Female coach. Maintained in laboratory kennels for several months under optimum conditions. Mar. 21, 1946—Blood protein depletion begun. Beginning weight 18.4 kilos, blood volume 1601 cc., plasma volume 809 cc., plasma protein 5.3 gm. per cent. Daily diet of protein-free basal biscuit 400 gm., canned carrots 150 gm., yeast 3 gm., liver extract powder 3 gm. Regular double depletion experiments with interspersed recovery period. Oct. 17, 1946—Daily diet of protein-free basal biscuit 450 gm., canned onions 100 gm., yeast 3 gm., liver extract powder 2 gm. Blood protein depletion begun. Beginning weight 18.6 kilos, blood volume 1534 cc., plasma volume 836 cc., plasma protein 5.3 gm. per cent.

Nov. 22—*Pig liver* (cooked) (Table 27). Daily diet of protein-free basal biscuit 500 gm., cooked pig liver 100 gm., yeast 3 gm., liver extract powder 2 gm. Beginning plasma volume 763 cc. Dec. 6—Protein-free basal biscuit increased to 400 gm., liver decreased to 70 gm. Dec. 13—Protein-free basal biscuit decreased to 300 gm., liver increased to 80 gm. Dec. 20—Liver increased to 90 gm. Dec. 22—Liver increased to 100 gm. Plasma volumes 815 cc., 810 cc., 810 cc., 764 cc., 728 cc. A/G ratios 1.6, 1.6, 1.3, 1.5, 1.5 (weekly intervals).

Dog 47-35 (Tables 24, 8).—

Male female coach. Maintained in laboratory kennels under optimum conditions. May 21, 1947—Daily diet of protein-free basal biscuit 500 gm., yeast 3 gm., liver extract powder 2 gm., canned carrots 150 gm., synthetic liquid vitamin mixture 10 cc. Beginning weight 18.4 kilos, blood volume 1534 cc., plasma volume 836 cc., plasma protein 5.3 gm. per cent. Regular double depletion experiments with interspersed recovery period.

July 11—*Peanut flour* (Rutgers University) (Table 24). Daily diet of protein-free basal biscuit 350 gm., peanut flour 65 gm., lard 15 gm., synthetic vitamin pills 3, dextrose 20 gm. July 30—Peanut flour decreased to 50 gm. Plasma volumes 610 cc., 572 cc., 550 cc., 522 cc., 511 cc. A/G ratios 1.2, 1.3, 1.1, 0.8, 0.9 (weekly intervals). Continued regular double depletion experiment.

Aug. 22—*Beef heart* (fresh) (Table 8). Daily diet of protein-free basal biscuit 350 gm., cooked beef heart 180 to 112 gm., lard 10 gm., synthetic vitamin pills 3. Beef heart intake varied. Plasma volumes 548 cc., 548 cc., 582 cc., 565 cc., 550 cc. A/G ratios 1.1, 1.6, 1.5, 1.3, 1.3 (weekly intervals).

EXPERIMENTAL OBSERVATIONS

The tabulated experiments deal with various egg fractions fresh and processed, fresh and processed meat, fresh beef heart, and canned salmon muscle. By and large the experiments are satisfactory and are of sufficient duration to be convincing. The difference between the effect of egg and beef muscle is quite evident whatever may be the correct explanation. More total new blood protein is produced on a meat diet and hemoglobin production greatly exceeds the plasma protein output. Egg materials favor the production of new plasma proteins but the total blood protein output falls below the levels due to the meat diet.

TABLE 1
Fresh Whole Egg

Weight	Protein intake		Food consumption	Protein output				Production ratio plasma protein to hemoglobin
	Type	Weekly		Hemoglobin		Plasma protein		
				Level	Output per wk.	Level	Output per wk.	
kg.		gm.	per cent	gm. per cent	gm.	gm. per cent	gm.	per cent
12.5	<i>Dog 45-6</i> Amino acid	194	96	12.4	52.6	4.5	16.6	
12.5	Whole egg	118	100	10.3	32.2	4.4	10.5	
12.1	Whole egg	147	86	8.9	14.9	4.5	5.8	
12.0	Whole egg	147	76	8.9	47.6	4.7	22.2	
11.8	Whole egg	166	96	6.6	20.4	5.0	15.3	
-0.7	Totals	578		(48.3)	115.1	(59.8)	53.8	120
11.4	<i>Dog 45-3</i> Liver	281	100	9.4	24.3	4.4	11.0	
11.4	Whole egg	199	98	8.7	22.7	4.5	10.2	
11.3	Whole egg	152	75	8.6	21.8	4.5	11.2	
11.2	Whole egg	203	100	5.5	27.7	4.9	19.5	
-0.2	Totals	554		(40.6)	72.2	(49.2)	40.9	120

In the tables figures in parentheses indicate "net corrected total output."

Tables 1 and 2 should be compared. The dogs were in good condition and weight changes are not significant—slight losses or gains—0.2, 0.5, and 0.7 kilo losses in 3 to 5 weeks and 1.0 kilo gain in 5 weeks. The plasma protein output was large and plasma to hemoglobin ratio high, 81 to 120 per cent. Table 1 illustrates the trend in these experiments—a rise in plasma protein levels and a fall in hemoglobin levels which call for corrections in protein output (based on blood volume) to give a true *net output*. The hemoglobin levels determine the amounts of blood which can safely be removed.

TABLE 2
Whole Egg (Rutgers University)

Weight	Protein intake		Food consumption	Protein output				Production ratio plasma protein to hemoglobin
	Type	Weekly		Hemoglobin		Plasma protein		
				Level	Output per wk.	Level	Output per wk.	
kg.		gm.	per cent	gm. per cent	gm.	gm. per cent	gm.	per cent
11.4	Dog 45-6 Lactalbumin	173	85	8.1	26.3	5.8	16.3	
11.4	Whole egg powder	166	79	8.4	21.3	5.5	13.7	
11.0	Whole egg powder	141	67	6.4	31.7	5.4	23.5	
10.8	Whole egg powder	158	75	6.9	10.7	5.7	7.9	
10.7	Whole egg powder	191	91	6.6	20.2	5.8	15.8	
10.9	Whole egg powder	210	100	7.0	22.1	5.9	15.3	
-0.5	Totals	866		(93.5)	106.0	(75.9)	76.2	81
12.6	Dog 45-2 Egg albumin	276	100	7.1	1.8	4.0	1.0	
12.7	Whole egg powder	240	100	8.2	1.7	4.4	1.1	
13.0	Whole egg powder	240	100	6.0	37.7	5.1	23.0	
13.0	Whole egg powder	240	100	7.5	11.6	5.1	9.1	
13.1	Whole egg powder	240	100	7.0	20.8	5.1	15.2	
13.6	Whole egg powder	240	100	6.0	21.6	5.6	16.2	
-1.0	Totals	1200		(59.2)	93.4	(51.3)	64.6	91

High egg albumin (Table 3) gave a response almost precisely that seen with whole fresh egg (Table 1). The weight was relatively constant (0.3 to 0.5 kilo loss in 4 and 5 weeks). The levels of hemoglobin fell, those of plasma protein rose, and the ratio of produced plasma protein to hemoglobin was high, 80 and 120 per cent.

TABLE 3
Fresh Egg Albumin

Weight	Protein intake		Food consumption	Protein output				Production ratio plasma protein to hemoglobin
	Type	Weekly		Hemoglobin		Plasma protein		
				Level	Output per wk.	Level	Output per wk.	
kg.		gm.	per cent	gm. per cent	gm.	gm. per cent	gm.	per cent
13.9	<i>Dog 40-36</i> Dried egg albumin	96	45	7.6	1.5	4.0	1.0	
13.7	Egg albumin	144	100	7.1	11.9	4.6	6.6	
13.6	Egg albumin	179	100	7.0	11.2	4.5	6.6	
13.3	Egg albumin	165	92	7.0	19.9	5.2	13.7	
13.4	Egg albumin	102	57	5.9	22.6	5.3	16.8	
-0.5	Totals.	590		(42.4)	65.6	(52.5)	43.7	120
15.6	<i>Dog 46-9</i> Pig stomach	219	100	7.4	20.6	4.7	11.5	
15.3	Egg albumin	162	100	8.6	11.9	4.6	6.6	
15.1	Egg albumin	172	100	6.4	38.2	5.4	22.2	
15.3	Egg albumin	179	100	6.9	13.0	5.1	8.3	
15.4	Egg albumin	187	100	7.5	21.3	5.5	15.3	
15.3	Egg albumin	187	100	7.1	26.0	5.5	18.1	
-0.3	Totals.	887		(96.1)	110.4	(76.1)	70.5	79

The findings with processed egg albumin (Table 4) differed from those with fresh albumin in these experiments. The cause for this difference is not clear but evidently the doubly depleted dog cannot use the processed albumin as effectively as it can the fresh egg albumin. The processing of the egg albumin (Bureau of Biological Research of Rutgers University) essentially includes a fermentation to remove the natural sugar. It is dried at a temperature not to exceed 65.5°C. and is kept as a fine powder which is readily reconstituted with water. The dogs in Table 4 all lost weight. The new blood protein formed was somewhat below the level noted for fresh egg albumin. The ratio of plasma to hemoglobin was low as compared with that recorded for fresh egg albumin. Loss of weight may have been in part responsible for some of the new formed blood protein. This would have made the protein production recorded as due to processed egg albumin somewhat greater than the true value.

The effect of fresh egg yolk (Table 5) was much like whole fresh egg but the preponderance of plasma protein was not observed in one of the experiments.

TABLE 4
Egg Albumin (Rutgers University)

Weight	Protein intake		Food consumption	Protein output				Production ratio plasma protein to hemoglobin
	Type	Weekly		Hemoglobin		Plasma protein		
				Level	Output per wk.	Level	Output per wk.	
kg.		grs.	per cent	grs. per cent	grs.	grs. per cent	grs.	per cent
14.1	Dog 45-2 Liver	235	100	8.5	46.0	4.6	18.4	
14.0	Egg albumin	214	100	7.9	38.0	4.7	19.0	
13.6	Egg albumin	214	100	8.0	9.4	4.6	4.8	
13.0	Egg albumin	245	100	8.8	28.2	4.6	13.4	
13.3	Egg albumin	245	100	6.8	22.0	4.3	11.2	
12.6	Egg albumin	276	100	7.1	1.8	4.0	1.0	
-1.5	Totals.....	1194		(74.4)	99.4	(42.5)	49.4	57
16.8	Dog 49-36 Basal	16	84	8.1	40.5	4.4	24.6	
16.5	Egg albumin	171	80	9.0	14.3	4.4	7.4	
15.8	Egg albumin	184	86	8.2	17.2	4.5	8.1	
15.3	Egg albumin	182	85	8.2	14.7	4.4	7.1	
14.6	Egg albumin	79	37	6.0	39.0	4.7	22.6	
13.9	Egg albumin	96	45	7.6	1.5	4.0	1.0	
-2.9	Totals.....	712		(64.9)	86.7	(36.3)	46.2	56
10.9	Dog 45-6 Whole egg powder	210	100	7.0	22.1	5.9	15.3	
10.6	Egg albumin	245	100	7.0	21.2	5.5	15.2	
10.1	Egg albumin	245	100	5.7	31.5	5.1	20.9	
-0.8	Totals	490		(39.8)	52.7	(30.0)	36.1	75

One dog gained 1.0, the other lost 0.7 kilo on practically an identical intake. This brought out the fact observed in many experiments that some animals are able to use or produce protein better than other animals, just as some animals can run faster than others. Obviously the past history of the dogs is important in evaluating the production of blood proteins or the dietary utilization of food proteins. The younger and heavier dog utilizes the egg yolk diet, gains weight, and produces a high average of blood proteins (Table 5).

Leaf meal, in contrast to egg showed a different picture (Table 6 and 7). The total output of new hemoglobin and plasma protein was almost double that

observed in the preceding experiments with egg. The hemoglobin production was much in excess of the plasma protein—almost 2 to 1. There was gain in weight in all experiments. The level of hemoglobin rose and the plasma protein levels fell or remained low and limited the bleeding to remove excess hemoglobin.

TABLE 5
Fresh Egg Yolk

Weight	Protein intake		Food consumption	Protein output				Production ratio plasma protein to hemoglobin
	Type	Weekly		Hemoglobin		Plasma protein		
				Level	Output per wk.	Level	Output per wk.	
kg.		gm.	per cent	gm. per cent	gm.	gm. per cent	gm.	per cent
13.6	<i>Dog 45-2</i> Whole egg powder	240	100	6.0	21.6	5.6	16.2	
13.8	Egg yolk	190	100	8.2	1.2	5.3	1.2	
14.6	Egg yolk	179	94	6.7	23.0	5.1	16.2	
14.8	Egg yolk	216	91	7.8	9.3	5.3	6.3	
14.6	Egg yolk	176	84	6.6	29.0	5.5	21.4	
14.6	Egg yolk	182	96	7.7	13.1	5.4	8.8	
+1.0	Totals.....	943		(98.2)	75.6	(53.5)	53.9	54
12.9	<i>Dog 44-16</i> Pig stomach	186	100	12.3	2.0	4.1	0.9	
12.8	Egg yolk	176	88	12.2	13.0	4.3	4.3	
12.5	Egg yolk	188	94	10.8	29.3	4.2	10.0	
12.6	Egg yolk	195	93	10.2	13.6	4.7	5.7	
12.6	Egg yolk	205	98	8.0	36.9	4.9	16.8	
12.2	Egg yolk	159	76	7.2	21.4	4.8	13.1	
-0.7	Totals.....	923		(57.7)	114.2	(57.9)	49.9	100

Beef muscle (processed) gave a picture like fresh beef. The muscle was heated to 82.2°C. and dried *in vacuo*. It was defatted with benzol and again dried *in vacuo* to remove all solvent. It was fed as a powder mixed in the basal ration (Table 7). There was slight gain in weight and the expected high output of hemoglobin and plasma protein. The predominance of new hemoglobin removed was great, 3 or 4 to 1.

Beef heart, fresh cooked (Table 8) had an effect but little different from that of beef skeletal muscle. The weight balance was positive. The total new

blood protein was less than with beef muscle but more than with egg. Preponderance of hemoglobin removed was noted.

TABLE 6
Fresh Beef Muscle

Weight	Protein intake		Food consumption	Protein output				Production ratio plasma protein to hemoglobin
	Type	Weekly		Hemoglobin		Plasma protein		
				Level	Output per wk.	Level	Output per wk.	
kg.		mg.	per cent	gm. per cent	gm.	gm. per cent	gm.	per cent
20.6	Dog 41-52 Lactalbumin	175	86	7.9	1.2	6.0	1.5	
20.8	Beef muscle	271	100	6.9	38.4	6.4	30.4	
21.4	Beef muscle	271	100	10.3	11.5	6.0	8.8	
21.8	Beef muscle	271	100	8.4	44.8	5.4	25.6	
22.1	Beef muscle	271	100	9.8	31.2	5.4	18.2	
21.9	Beef muscle	271	100	8.6	62.5	5.3	34.4	
22.5	Beef muscle	252	93	9.5	45.6	5.3	27.7	
+1.9	Totals.....	1607		(265.0)	234.0	(134.9)	145.1	51
16.1	Dog 42-1 Liver	227	88	7.3	33.2	5.0	19.5	
16.3	Beef muscle	244	95	8.8	23.5	5.5	16.5	
16.4	Beef muscle	257	100	8.6	36.4	5.2	22.9	
16.7	Beef muscle	257	100	7.8	36.0	5.1	22.6	
16.3	Beef muscle	248	96	8.1	38.3	5.2	23.6	
+0.2	Totals.....	1006		(146.5)	134.2	(88.0)	85.6	60
12.6	Dog 44-16 Soy bean	176	70	11.2	2.3	4.0	1.0	
12.6	Beef muscle	249	100	11.8	33.8	4.4	10.6	
13.0	Beef muscle	248	100	10.3	41.2	4.5	14.0	
13.0	Beef muscle	249	100	11.1	37.9	4.6	14.9	
13.3	Beef muscle	250	100	11.8	29.3	4.6	11.2	
13.1	Beef muscle	250	100	12.3	24.0	4.4	7.8	
+0.5	Totals.....	1246		(184.0)	166.2	(61.2)	58.5	33

Soy bean muscle gave a picture (Table 9) almost exactly like that observed with beef heart. The total nitrogen figures are not given in the tables but analyses were made in all experiments to record urinary nitrogen. The total

nitrogen output includes fecal nitrogen which was not determined but in similar experiments it ran about 2.0 gm. per week. The removed nitrogen of the plasma and hemoglobin can readily be calculated. The total nitrogen balance was strongly positive in all experiments tabulated.

TABLE 7
Beef Muscle (Rutgers University)

Weight	Protein intake		Food consumption	Protein output				Production ratio plasma protein to hemoglobin
	Type	Weekly		Hemoglobin		Plasma protein		
				Level	Output per wk.	Level	Output per wk.	
kg.		gm.	per cent	gm. per cent	gm.	gm. per cent	gm.	per cent
12.6	Dog 46-10 Basal	17	88	10.0	13.6	4.4	5.6	
12.4	Beef muscle	235	94	12.2	2.6	4.0	1.0	
13.0	Beef muscle	250	100	10.9	33.1	4.6	10.8	
12.8	Beef muscle	250	100	11.2	35.9	4.6	11.9	
13.2	Beef muscle	250	100	9.4	46.1	4.6	17.4	
13.3	Beef muscle	210	84	11.0	35.7	4.5	12.6	
+0.7	Totals.....	1195		(174.8)	153.4	(56.6)	53.7	32
14.6	Dog 44-16 Beef heart	244	100	8.8	38.4	5.0	18.0	
14.7	Beef muscle	250	100	11.6	24.0	4.5	10.8	
14.1	Beef muscle	250	100	10.8	44.0	4.6	16.0	
14.4	Beef muscle	250	100	12.3	33.4	4.5	12.0	
14.5	Beef muscle	250	100	12.2	48.5	4.5	15.8	
14.2	Beef muscle	240	96	12.5	36.2	4.6	12.0	
-0.4	Totals.....	1240		(226.2)	186.1	(60.3)	66.6	27

Summary Table 10 gives the general picture of these experiments. It is obvious that much more total protein (hemoglobin and plasma protein) is produced on a meat diet than on an egg diet of comparable amount. In general the egg diet favors plasma protein production when compared with meat which favors hemoglobin production. Processed egg albumin as used was not as well utilized by the dog, weight loss is observed, and blood protein output is modified. Body weight loss is a factor in the response to processed egg albumin as it is recognized that the doubly depleted dog conserves materials which con-

TABLE 8
Fresh Beef Heart

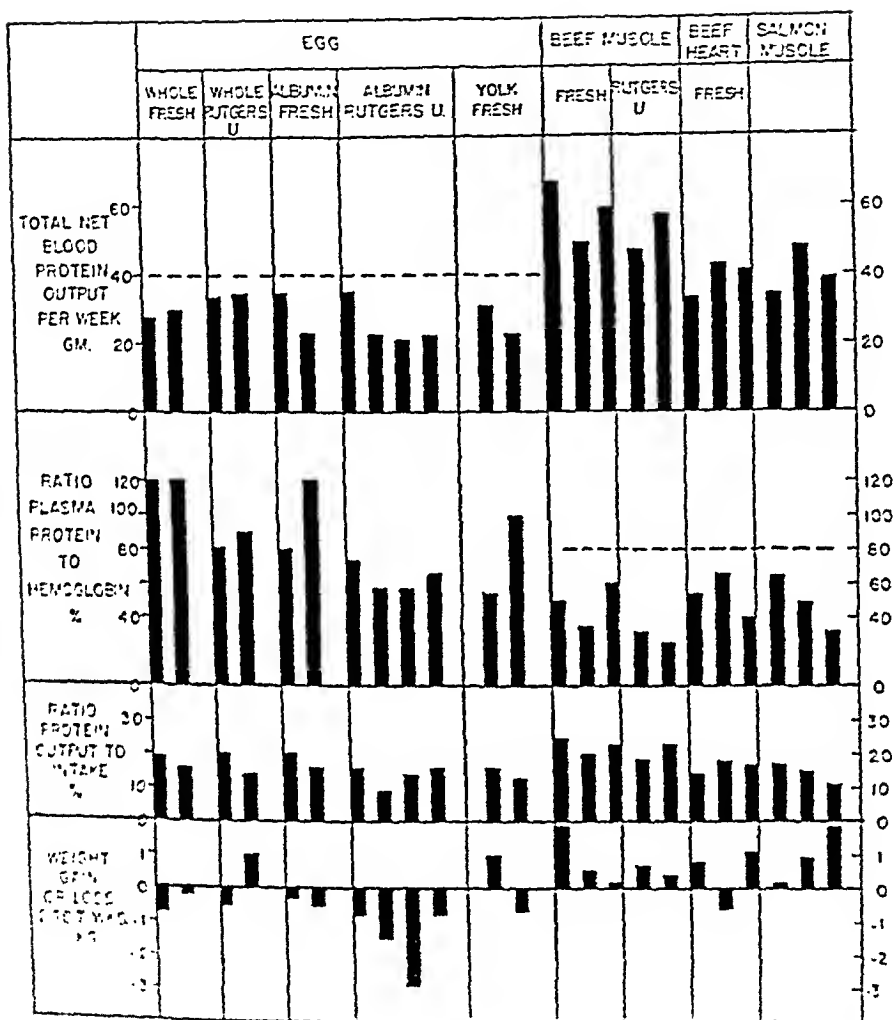
Weight	Protein intake		Food consumption	Protein output				Production ratio plasma protein to hemoglobin
	Type	Weekly		Hemoglobin		Plasma protein		
				Level	Output per wk.	Level	Output per wk.	
kg.		gm.	per cent	gm. per cent	gm.	gm. per cent	gm.	per cent
10.2	<i>Dog 46-5</i> Peanut flour	100	90	11.8	2.0	4.7	1.0	
10.3	Beef heart	244	100	8.8	29.9	4.6	10.3	
10.3	Beef heart	244	100	9.7	24.2	4.6	11.5	
10.6	Beef heart	207	85	7.2	32.6	4.4	12.5	
11.0	Beef heart	226	93	8.5	14.1	4.4	7.2	
11.0	Beef heart	208	85	9.3	17.7	4.9	9.4	
+0.8	Totals	1129		(104.8)	118.5	(56.9)	50.9	54
15.2	<i>Dog 44-16</i> Casein	222	100	13.6	34.4	4.7	11.2	
15.0	Beef heart	242	99	12.3	35.1	4.3	10.1	
14.7	Beef heart	244	100	10.2	45.9	4.9	18.2	
14.7	Beef heart	244	100	8.8	43.9	4.7	18.5	
14.9	Beef heart	244	100	8.7	33.9	4.8	16.7	
14.6	Beef heart	244	100	8.8	38.4	5.0	18.0	
-0.6	Totals	1218		(127.5)	197.2	(86.4)	81.5	67
10.5	<i>Dog 47-25</i> Fibrin	172	70	9.0	26.5	4.5	10.1	
11.0	Beef heart	245	100	10.4	18.9	4.6	8.7	
10.4	Beef heart	242	99	8.8	34.9	4.6	16.5	
11.1	Beef heart	208	100	8.4	32.9	4.6	17.3	
11.3	Beef heart	222	100	9.8	14.6	4.7	7.1	
11.6	Beef heart	242	100	10.3	22.1	4.6	8.0	
-1.1	Totals	1159		(145.0)	123.4	(59.2)	57.6	41

tribute to new hemoglobin and plasma proteins during periods of weight loss— and loss of body protein stores (5). Beef heart and salmon muscle are alike and follow the general pattern response of beef muscle—perhaps a little less effectively and tend to produce more hemoglobin and plasma protein.

TABLE 9
Canned Salmon Muscle

Weight	Protein intake		Food consumption	Protein output				Production ratio plasma protein to hemoglobin
	Type	Weekly		Hemoglobin		Plasma protein		
				Level	Output per wk.	Level	Output per wk.	
kg.		gm.	per cent	gm. per cent	gm.	gm. per cent	gm.	per cent
13.5	<i>Dog 40-36</i> Amino acid	143	41	5.7	13.1	4.9	6.5	
14.0	Salmon	254	96	8.7	25.5	4.6	12.6	
14.1	Salmon	265	100	8.2	26.4	4.4	11.9	
14.1	Salmon	298	92	6.6	34.5	4.6	19.6	
14.0	Salmon	292	90	6.9	10.6	4.4	5.9	
14.3	Salmon	411	100	8.0	36.1	5.0	21.5	
14.3	Salmon	411	100	7.8	37.2	5.0	22.7	
14.4	Salmon	345	85	8.5	23.5	5.0	15.7	
+0.9	Totals.....	2276		(225.5)	193.8	(110.0)	109.9	49
15.4	<i>Dog 40-34</i> Amino acid	136	18	6.2	1.3	6.3	1.0	
17.2	Salmon	352	100	7.7	24.6	5.2	16.2	
17.1	Salmon	352	100	7.1	23.0	5.1	15.2	
17.2	Salmon	352	100	8.9	20.7	5.1	12.5	
17.3	Salmon	352	100	8.9	13.8	5.2	7.0	
+1.9	Totals.....	1408		(119.0)	82.1	(39.1)	50.9	33
11.0	<i>Dog 46-5</i> Beef heart	208	85	9.3	17.7	4.9	9.4	
11.3	Salmon	245	100	7.0	38.6	4.9	21.6	
11.5	Salmon	196	80	6.8	23.0	4.7	12.2	
11.2	Salmon	213	87	7.1	20.7	4.6	12.1	
11.1	Salmon	169	69	6.9	19.2	4.8	12.0	
11.1	Salmon	218	74	7.2	19.9	4.6	11.8	
+0.1	Totals.....	1041		(103.6)	121.4	(69.0)	69.7	66

SUMMARY TABLE 10



SUMMARY

Doubly depleted dogs (anemic and hypoproteinemic) respond favorably to all the diet proteins used in the above experiments.

Egg products (whole egg, albumin, or egg yolk) are well utilized by these dogs. Egg proteins favor the production of plasma protein and in some experiments the output of plasma protein is actually more than the output of hemoglobin. In contrast fresh beef muscle favors hemoglobin production—the output being 3 or 4 times that of plasma protein.

The processed egg albumin fed in Table 4 was not well utilized and there was weight loss.

Beef muscle (fresh or processed) gives a *total* blood protein output about twice that with egg feeding and there is a striking preponderance of hemoglobin output.

Beef heart and salmon muscle show a pattern much like beef muscle. The total blood protein output is below that due to beef muscle.

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DIETARY EFFECTS ON ANEMIA PLUS HYPOPROTEINEMIA IN DOGS

I. THE FINDINGS WITH MILK PRODUCTS, WHEAT, AND PEANUT FLOURS AS COMPARED WITH LIVER*

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When we compare the effect of the proteins in milk products, wheat and peanut flour, and liver with that of the proteins tabulated in Paper I (1), there are significant differences. *Casein* purified or as a commercial powder is a superior protein as promoting new hemoglobin and plasma protein output. *Casein* in fact compares favorably with liver and meat under the experimental conditions. *Lactalbumin* by contrast is not as effective in promoting blood protein output and tends to favor plasma protein production, in this respect resembling egg albumin. *Peanut flour* is definitely inadequate to promote satisfactory new hemoglobin and plasma protein output. *Wheat gluten* is neither superior nor inferior to many proteins but is difficult to feed over many weeks and distaste limits food intake, causes weight loss, and complicates the observations. *Liver* serves as a control as its capacity to promote hemoglobin and plasma protein production is well established.

The details of method and the experimental histories are given in Paper I.

EXPERIMENTAL OBSERVATIONS

Casein purified for the Bureau of Biological Research of Rutgers University was tested in three adequate experiments, given in Table 21. The casein was precipitated by acid and washed with suitable acid water solutions at the proper pH. No solvents were used. Drying was done in warm air (60–70°C.) and material was ground in a hammer mill. All experiments show complete food consumption and essential weight balance during 4 and 5 week diet periods. The blood protein output was very high, 45 to 70 gm. per week, which corresponds to beef muscle feeding. The ratio of hemoglobin production was high (3 or even 4 times the plasma protein) and the ratio of blood protein output to food protein intake was also high (26 to 50 per cent).

Commercial casein purchased in the open market was also tested (Table 22). The response was much like that noted with pure casein (Table 21). The total

* We are indebted to E. Lilly and Company for aid in conducting this work. We are indebted to Dr. James E. Allison, Dr. William H. Cole, and Dr. E. H. Lamm of Rutgers University who aided this cooperative investigation in evaluating protein foods.

TABLE 21

Casein (Rulgers University)

Weight	Protein intake		Food consumption	Protein output				Production ratio plasma protein to hemoglobin
	Type	Weekly		Hemoglobin		Plasma protein		
				Level	Output per wk.	Level	Output per wk.	
kg.		gm.	per cent	gm. per cent	gm.	gm. per cent	gm.	per cent
<i>Dog 45-2</i>								
14.9	Basal	19	100	8.6	61.5	4.6	24.4	
14.9	Casein	208	100	9.3	35.4	4.5	16.5	
14.9	Casein	237	100	10.7	47.4	4.9	19.6	
14.4	Casein	237	100	10.5	53.7	5.2	21.9	
14.8	Casein	237	100	10.4	44.9	4.9	19.8	
14.6	Casein	237	100	11.0	45.3	4.9	18.5	
-0.3	Totals.....	1156		(255.1)	226.7	(95.9)	96.3	38
<i>Dog 45-6</i>								
11.8	Whole egg	166	96	6.6	20.5	5.0	15.3	
11.9	Casein	208	100	9.7	1.9	5.0	1.6	
11.8	Casein	208	100	10.2	48.1	4.7	16.9	
11.7	Casein	208	100	11.2	45.0	4.8	17.1	
11.7	Casein	208	100	10.7	42.4	4.7	15.4	
11.7	Casein	208	100	12.0	43.0	4.9	16.4	
-0.1	Totals.....	1040		(248.6)	180.4	(64.7)	67.4	26
<i>Dog 42-1</i>								
17.0	Lactalbumin	173	100	5.9	19.9	5.2	15.0	
17.2	Casein	178	100	8.0	1.5	5.6	1.0	
17.1	Casein	178	100	8.1	28.8	5.4	17.5	
16.9	Casein	173	97	8.6	37.7	5.2	21.5	
17.0	Casein	160	90	10.5	21.1	4.8	11.2	
0	Totals.....	689		(143.3)	89.1	(39.5)	51.2	28

In the tables figures in parentheses indicate "net corrected total output."

weekly output of hemoglobin and plasma protein was 53 and 67 gm. One dog lost 0.7 kilo in 5 weeks but this was a large animal (22 kilos). This dog showed a higher ratio of plasma protein to hemoglobin than the others on a casein diet. Both dogs ate all their diet and were in perfect condition.

Lactalbumin (Table 23) showed significant differences when compared with

casein. This was a commercial product. One dog had a slight weight gain, one a slight weight loss, and a third considerable weight loss in a 5 week experiment. The total weekly blood protein output was lower than that with casein—31 to 57 gm. and the ratio of protein output to food protein intake was also lower. The most striking difference was in the ratio of plasma protein to

TABLE 22
Casein (Commercial)

Weight	Protein intake		Food consumption	Protein output				Production ratio plasma protein to hemoglobin
	Type	Weekly		Hemoglobin		Plasma protein		
				Level	Output per wk.	Level	Output per wk.	
kg.		gm.	per cent	gm. per cent	gm.	gm. per cent	gm.	per cent
22.7	<i>Dog 40-32</i> Fibrin	245	100	8.3	2.5	5.8	1.4	
22.8	Casein	222	100	7.8	27.6	5.5	17.0	
22.4	Casein	222	100	9.4	39.7	6.3	26.2	
22.1	Casein	222	100	7.5	42.9	6.4	28.1	
22.3	Casein	222	100	9.2	26.4	6.2	20.3	
22.0	Casein	222	100	9.5	45.6	6.6	33.3	
-0.7	Totals.....	1110		(199.7)	182.2	(133.8)	124.9	67
15.3	<i>Dog 44-16</i> Basal	18	97	10.9	25.5	4.3	9.8	
14.9	Casein	222	100	10.7	27.3	4.1	9.5	
14.4	Casein	222	100	12.0	26.9	4.2	8.5	
14.3	Casein	222	100	13.2	31.4	4.4	10.9	
14.7	Casein	222	100	11.4	47.0	4.6	16.0	
15.2	Casein	222	100	13.6	34.4	4.7	11.2	
-0.1	Totals.....	1110		(205.5)	167.0	(57.6)	56.1	28

hemoglobin which was high—72, 90, and 94 per cent. One notes that in all experiments the hemoglobin levels fell and the plasma protein levels rose as was noted in the experiments with whole egg and egg albumin. *Lactalbumin* seems to favor plasma protein production in contrast to casein and liver (Table 27).

Peanut flour, purified or commercial, (Tables 24 and 25) did not support much red blood protein production. Peanut flour was prepared for the Bureau of Biological Research of Rutgers University using the McMath-Horand process. This gave a partially defatted flour; analyses will be reported elsewhere. The findings with processed flour differed in no significant

TABLE 23
Lactalbumin (Commercial)

Weight	Protein intake		Food consumption	Protein output				Production ratio plasma protein to hemoglobin
	Type	Weekly		Hemoglobin		Plasma protein		
				Level	Output per. wk.	Level	Output per wk.	
kg.		gm.	per cent	gm. per cent	gm.	gm. per cent	gm.	per cent
14.6	<i>Dog 45-2</i> Casein	237	100	11.0	45.3	4.9	18.5	
14.8	Lactalbumin	239	100	9.0	63.9	4.9	24.3	
15.3	Lactalbumin	239	100	6.9	49.9	5.1	28.8	
15.0	Lactalbumin	225	94	7.3	22.5	5.3	14.6	
15.3	Lactalbumin	239	100	7.4	25.0	5.6	14.9	
15.4	Lactalbumin	239	100	7.7	38.9	5.6	25.0	
+0.8	Totals.....	1181		(166.7)	200.2	(119.7)	107.6	72
11.7	<i>Dog 45-6</i> Casein	208	100	12.0	43.0	4.9	16.4	
11.5	Lactalbumin	148	73	8.3	60.4	4.8	22.3	
11.5	Lactalbumin	124	61	8.3	21.0	4.8	12.1	
11.2	Lactalbumin	144	71	5.7	28.2	5.1	19.2	
11.6	Lactalbumin	195	96	7.9	1.7	5.3	1.3	
11.4	Lactalbumin	173	85	8.1	26.3	5.8	16.3	
-0.3	Totals.....	784		(80.6)	137.6	(75.5)	71.2	94
14.1	<i>Dog 46-4</i> Fibrin	228	93	8.3	27.8	5.5	16.4	
14.0	Lactalbumin	207	85	7.7	35.5	5.2	22.6	
13.7	Lactalbumin	173	71	8.6	21.9	5.4	15.3	
12.9	Lactalbumin	110	45	6.8	20.8	5.7	16.0	
12.6	Lactalbumin	150	80	7.7	19.9	6.0	14.1	
11.7	Lactalbumin	147	83	6.0	28.6	5.4	21.4	
-2.4	Totals.....	787		(95.7)	126.7	(86.2)	89.4	90

manner from those with the commercial product when it was incorporated in these diets.

The peanut flour was not well eaten and distaste for the food mixture terminated some experiments not reported here, and shortened others. Peanut flour was incorporated in baked biscuit with no increase in palatability. All dogs

lost weight—1 to 2 kilos in 3 to 5 weeks. Only one dog tolerated the diet for 5 weeks and this dog, 47-25, (Table 24) gave the highest output of blood protein per week—33 gm. A glance at the table however shows that the peanut flour experiment followed a casein experiment with no basal diet period intervening. There was obviously some "carry-over" from the very favorable casein diet,

TABLE 24
Peanut Flour (Rutgers University)

Weight	Protein intake		Food consumption	Protein output				Production ratio plasma protein to hemoglobin
	Type	Weekly		Hemoglobin		Plasma protein		
				Level	Output per wk.	Level	Output per wk.	
kg.		gm.	per cent	gm. per cent	gm.	gm. per cent	gm.	per cent
11.8	Dog 47-25 Casein	242	100	9.3	34.0	5.0	18.0	
11.8	Peanut flour	239	100	8.8	41.4	5.2	21.9	
11.5	Peanut flour	220	92	10.2	24.3	4.7	11.4	
11.0	Peanut flour	174	78	9.7	23.6	4.8	9.7	
10.7	Peanut flour	123	67	10.9	14.5	4.3	5.5	
10.7	Peanut flour	147	80	11.1	2.4	4.5	0.8	
-1.1	Totals.....	903		(122.5)	106.2	(44.5)	49.3	36
11.6	Dog 46-5 Basal	19	100	8.7	40.2	4.4	17.4	
11.1	Peanut flour	134	91	8.7	19.6	4.2	8.8	
10.5	Peanut flour	113	77	11.2	2.0	4.3	1.2	
10.2	Peanut flour	100	90	11.8	2.0	4.7	1.0	
-1.4	Totals.....	347		(40.3)	23.6	(7.4)	11.0	18

and the 1st week showed very high output figures for hemoglobin and plasma protein—a surplus of about 30 gm. due to the casein carry-over. This would reduce the *true weekly output* from 33.7 gm. to approximately 28 gm. The total blood protein output in all experiments did not amount to 50 per cent of the blood protein output due to casein feeding. The ratio of blood protein production to food protein intake was very low. The ratio of plasma protein to hemoglobin was low in all experiments except dog 41-53 (Table 25). Here egg feeding preceded the peanut flour diet, and as there was no basal diet interval there probably was some "carry-over" with more plasma protein produced

(egg favors plasma protein over hemoglobin). This may in part explain the high plasma protein to hemoglobin ratio.

TABLE 25
Peanut Flour (Commercial)

Weight	Protein intake		Food consumption	Protein output				Production ratio plasma protein to hemoglobin
	Type	Weekly		Hemoglobin		Plasma protein		
				Level	Output per wk.	Level	Output per wk.	
kg.		gm.	per cent	gm. per cent	gm.	gm. per cent	gm.	per cent
19.3	Dog 41-53 Basal	23	90	5.9	24.0	5.0	15.6	
18.7	Peanut flour	198	84	6.3	1.4	5.4	1.0	
18.4	Peanut flour	221	94	6.6	42.6	5.0	23.2	
17.6	Peanut flour	182	77	6.6	1.9	4.5	1.2	
-1.7	Totals.....	601		(49.3)	45.9	(16.9)	25.4	34
16.5	Dog 41-53 Egg	160	76	8.0	21.3	5.4	13.2	
16.2	Peanut flour biscuit*	181	75	5.6	32.3	5.0	18.2	
15.2	Peanut flour biscuit*	253	83	5.6	7.6	4.4	5.1	
14.6	Peanut flour biscuit*	276	91	6.9	22.5	4.8	12.4	
-1.9	Totals.....	710		(43.5)	62.4	(29.0)	35.7	67

* Baked into biscuit.

Wheat gluten (Table 26) was not well suited to this type of experiment and the dogs usually ate the mixture with reluctance. Wheat gluten as prepared for the Bureau of Biological Research of Rutgers University represented wheat flour treated with cold water to hydrate the gluten. Water was added repeatedly in further treatment to remove the starch and the washings were at a temperature of 90—170°F. Finally the gummy matrix was dried *in vacuo* in trays at 130°F. No defatting was attempted.

The experiments with *wheat gluten* show differences which we believe are in large part explained by weight loss. One dog (45-3, Table 26) ate the diet for 4 weeks and actually gained 0.1 kilo. This dog showed a low average figure (34 gm.) for total net weekly blood protein output. The two other dogs of Table 26 showed rapid weight loss (3.0 and 2.8 kilos) in 4 weeks and a lower wheat gluten intake because of distaste for this food. This weight loss we assume explains some of the increase in net figures for whole blood protein

TABLE 26
Wheat Gluten (Rutgers University)

Weight	Protein intake		Food consumption	Protein output				Production ratio plasma protein to hemoglobin
	Type	Weekly		Hemoglobin		Plasma protein		
				Level	Output per wk.	Level	Output per wk.	
kg.		gm.	per cent	gm. per cent	gm.	gm. per cent	gm.	per cent
21.7	Dog 42-1 Basal	19	100	9.8	39.9	4.9	20.1	
20.5	Wheat gluten	173	84	10.1	41.2	4.6	18.7	
19.8	Wheat gluten	182	95	8.7	54.4	5.0	27.3	
19.0	Wheat gluten	155	72	8.7	27.1	4.9	14.0	
18.7	Wheat gluten	188	85	9.6	26.8	4.5	11.8	
-3.0	Totals.....	698		(125.6)	149.5	(60.2)	71.8	48
12.6	Dog 45-3 Basal	31	100	11.0	30.0	4.2	10.7	
12.9	Wheat gluten	244	100	12.8	2.0	4.1	1.0	
12.9	Wheat gluten	207	98	12.3	31.0	4.4	8.4	
13.1	Wheat gluten	202	95	12.0	33.3	4.5	9.9	
12.7	Wheat gluten	201	94	10.1	42.7	4.4	15.7	
+0.1	Totals.....	854		(96.8)	109.0	(37.4)	35.0	39
21.9	Dog 40-32 Amino acid, casein	211	100	9.3	36.4	4.6	17.1	
20.7	Wheat gluten	165	76	8.2	59.1	4.9	26.8	
20.0	Wheat gluten	115	67	9.8	43.4	5.2	20.8	
19.7	Wheat gluten	168	84	7.8	45.9	5.2	23.9	
19.1	Wheat gluten	212	98	8.8	36.0	4.7	19.3	
-2.8	Totals.....	660		(153.7)	184.4	(85.6)	90.8	55

output (60 and 47 gm.) as the body conserves material under conditions of weight loss which contribute to new blood proteins (2). The same explanation holds for the high ratios of protein output to intake (Summary Table 28).

Pig liver (Table 27) is a standard diet factor and we have completed many such experiments. The three given are fair examples. There were trivial weight fluctuations in the first two experiments and gain of 1.0 kilo in the third experiment. Production of hemoglobin was conspicuous and exceeded the plasma protein output three to one. The hemoglobin level in the circulating

blood rose in spite of heavy bleeding. The weekly net output of hemoglobin and plasma protein was 45, 48, and 53 gm.

TABLE 27

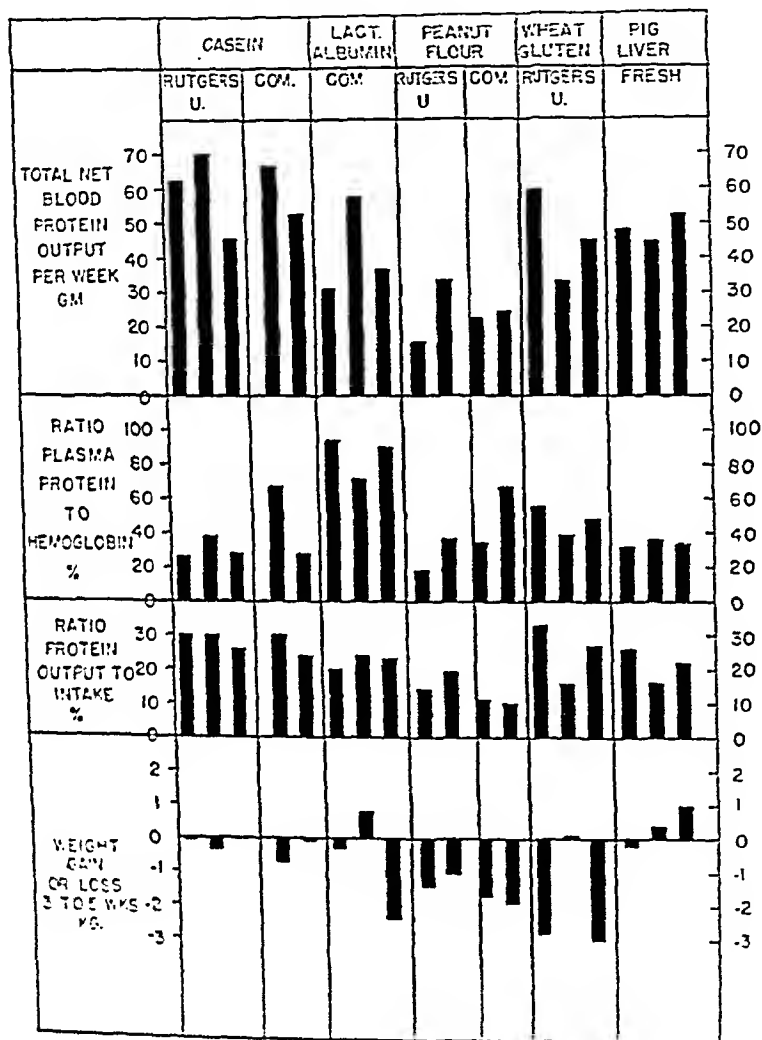
Pig Liver

Weight	Protein intake		Food consumption	Protein output				Production ratio plasma protein to hemoglobin
	Type	Weekly		Hemoglobin		Plasma protein		
				Level	Output per wk.	Level	Output per wk.	
kg.		gm.	per cent	gm. per cent	gm.	gm. per cent	gm.	per cent
11.0	<i>Dog 45-3</i> Amino acids	169	68	7.7	22.4	4.6	10.6	
11.1	Liver	261	98	9.7	13.8	4.2	6.4	
11.2	Liver	281	100	8.3	44.0	4.7	20.3	
11.4	Liver	281	100	9.4	24.3	4.4	11.0	
+0.4	Totals.....	823		(98.4)	82.1	(35.3)	37.7	36
15.5	<i>Dog 46-22</i> Amino acids	155	48	10.3	29.6	4.4	12.5	
15.9	Liver	239	98	10.1	24.2	4.5	10.1	
15.8	Liver	234	96	9.2	43.5	4.7	16.8	
15.6	Liver	102	65	9.7	27.5	4.4	10.8	
15.1	Liver	153	85	10.3	28.2	4.5	10.6	
15.3	Liver	202	100	11.9	32.4	4.4	11.5	
-0.2	Totals.....	930		(182.0)	155.8	(58.2)	59.8	32
16.3	<i>Dog 46-9</i> Basal	19	100	8.9	67.5	4.6	22.5	
16.3	Liver	247	100	10.6	31.2	4.5	12.4	
16.4	Liver	247	100	8.3	49.2	4.7	20.2	
16.6	Liver	247	100	9.5	26.9	4.8	12.1	
16.7	Liver	240	100	10.7	30.6	4.5	11.8	
17.3	Liver	239	100	10.4	30.2	4.4	10.8	
+1.0	Totals.....	1220		(196.6)	168.1	(66.6)	67.3	34

Summary Table 28 gives comparative values for these various proteins. *Casein* and *liver* stand out as most efficient. Both give maximal amounts of new formed blood protein (hemoglobin and plasma protein), if anything casein somewhat more than whole liver. The ratio of hemoglobin to plasma protein is about 3 to 1 with both pure and commercial casein and whole liver. The ratio

of blood protein output to food protein intake is correspondingly high. Weight is constant, food consumption adequate, and condition of the dogs excellent.

SUMMARY TABLE 28



Peanut flour, purified or commercial, stands in striking contrast to casein. The new blood protein produced is about one-half that following casein feeding. Plasma protein to hemoglobin ratio ranges from 20 to 67 per cent. The ratio of blood protein output to food protein intake is low. There is some weight loss in all experiments and the food mixtures are distasteful to dogs.

Lactalbumin is not as favorable for new blood protein production as is casein but the lactalbumin definitely favors plasma protein production and the ratio of plasma protein to hemoglobin is high (72, 90, and 94 per cent).

Wheat gluten is better than peanut flour but is not in the class with casein. The ratio of plasma protein to hemoglobin runs 40 to 55 per cent. There is conspicuous loss of weight (food distasteful) which in part is responsible for the apparently high levels of total blood protein production in two experiments.

SUMMARY

Casein (purified or commercial) in this type of experiment falls in the top bracket as a protein consistently favorable for maximal new hemoglobin and plasma protein production in doubly depleted dogs (anemic and hypoproteinemic).

Lactalbumin is less favorable for total blood protein production and the ratio of plasma protein to hemoglobin is high—that is lactalbumin favors plasma protein production as compared with casein, or is less favorable for hemoglobin production.

Peanut flour (purified or commercial) is less than half as effective as casein in promoting new blood protein production. The ratio of plasma protein to hemoglobin is about the same as casein.

Wheat gluten as tested is distasteful to dogs. It is neither very good nor very poor for blood protein production when it is eaten. There is nothing unusual about the response. Weight loss usually confuses the picture.

Liver stands as a control base line for the above experiments. Its capacity to further hemoglobin and plasma protein production is well established. The production of hemoglobin was about 3 times that of plasma protein in the experiments.

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IMMOBILIZATION OF *TREPONEMA PALLIDUM* IN VITRO BY ANTIBODY PRODUCED IN SYPHILITIC INFECTION*

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It is well established that animals and human beings infected with *Treponema pallidum* become resistant to reinfection with the same organism, but the mechanism of this immunity is poorly understood. One of the serious handicaps to studies on this problem has been the lack of *in vitro* methods for the detection of antibody to *T. pallidum*.

Early reports of an *in vitro* spirocheticidal action of serum and spinal fluid from patients in the late stages of syphilitic infection (1-3) were based on crude qualitative tests and did not offer experimental data adequate to establish the claims made. Such findings were not confirmed by others (4, 5) and recent experiments in this laboratory (10) did not yield convincing evidence of an *in vitro* effect.

The presence of spirocheticidal antibody in serum from syphilitic individuals has been demonstrated by animal "protection" tests (6-10), but the costly and cumbersome nature of these tests, as well as their qualitative character, renders them unsuitable for systematic investigation of the rôle and mechanism of specific humoral immunity in syphilis.

Since it is now agreed that *T. pallidum* has not as yet been cultivated and is therefore not available in adequate amounts for the usual *in vitro* immunological studies, various cultivatable, non-pathogenic spirochetes have been used in agglutination and complement-fixation tests (11-25) with sera from syphilitic animals and human beings. However, a considerable proportion of presumably normal sera has been found to react with these antigens, though usually in low titre. This reactivity with normal sera, as well as the lack of a clear understanding of the relationship of these non-virulent spirochetes to pathogenic *T. pallidum*, renders their use as antigens in the study of humoral immunity in syphilis of dubious value.

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While standard serological tests for syphilis, carried out with lipoidal antigens from beef heart or other mammalian tissues, serve to detect the presence of syphilitic infection with a relatively high degree of specificity, they do not provide an index of immunity to the disease. This is apparent from the decline in the titre of Wassermann antibody, or "reagin," with progression of the disease into the latent stage, although it is during this stage that animals exhibit a high resistance to reinfection (26, 27). Furthermore, it is questionable whether the appearance of reagin represents a specific immunological response to an antigenic constituent of *T. pallidum* (28-35). Thus, studies on immunity to syphilis have been restricted to *in vivo* experiments involving either active or passive "protection" tests.

As a result of the development in this laboratory of techniques which permit the extraction of virulent *T. pallida* from rabbit testicular syphilomas in a relatively tissue-free state and the maintenance of these organisms *in vitro* in a highly active state for several days (36), it has become possible to demonstrate the presence of an antibody in sera from syphilitic animals and human beings, which immobilizes virulent *T. pallida in vitro*.

The present report is concerned with: (a) the demonstration that this immobilizing activity is due to specific antibody acting in conjunction with complement, and that immobilized treponemes are non-infectious and presumably dead; (b) a study of factors which affect the measurement of the immobilizing antibody in sera from syphilitic individuals; (c) the occurrence of this antibody in sera from animals and human beings in various stages of syphilitic infection; and (d) the differentiation of the immobilizing antibody from the Wassermann antibody or reagin.

Materials and Techniques

Preparation of Treponeme Suspensions.—The general procedures for the collection and maintenance of virulent *T. pallidum in vitro*, are described and discussed in detail in a preceding paper from this laboratory (36).

In brief, rabbits to be used as source animals are inoculated intratesticularly with 0.5 ml. of a suspension of *T. pallida* freshly isolated from rabbit testicular syphilomas. These suspensions are prepared by Waring blender emulsification of 2 testicular syphilomas with approximately 15 ml. of a 5 per cent solution of crystalline bovine albumin in saline, and contain about 5 to 10×10^7 treponemes per ml. The animals are caged in an air-conditioned room (16-18°C.) and are examined daily for the development of syphilitic orchitis. After 7 to 14 days a recognizable orchitis becomes manifest, during the first 48 hours of which the animal is exsanguinated and the testes are removed. Following removal, the testes are immediately cut into thin slices with a specially constructed plastic instrument¹ fitted with 10 razor blades, and

¹ Constructed by Acme Metal Products, Inc., Baltimore. The instrument measures $5 \times 9 \times 2$ cm. and consists of 2 separate parts. The base has one deep transverse depression into which the rabbit testes can be placed securely, and 10 narrow longitudinal grooves. The upper

then washed with chilled 0.85 per cent saline to remove loose tissue particles. The slices are placed in 34 ml. of basal medium² under an atmosphere of 5 per cent carbon dioxide and 95

part is fitted with 10 Durham Duplex razor blades, held in parallel approximately 5 mm. apart. After the testes are placed in the transverse depression in the base the upper part may be placed on the base with the blades fitting into the longitudinal grooves and the testis cut into 8 to 10 slices of approximately 5 mm. width each by a downward and backward slicing movement.

² The constituents of the basal medium are made up individually and mixed in proper proportion just prior to use. The method of preparation and amounts used to make 85 ml. of medium are listed below. In previous experiments, these amounts were put into 100 ml. of medium to yield concentrations optimal for survival (36). In order to achieve the same concentrations in the present immobilization test mixtures, the same amounts were dissolved in 85 ml. since the constituents of the basal medium are diluted by the factor 85/100 when 1.7 ml. of medium containing treponemes is mixed with 0.2 ml. of serum and 0.1 ml. of guinea pig complement.

	Final concentration in test mixture
Crystalline bovine albumin.....	0.00028 M
5 gm. are dissolved in about 95 ml. of 0.85 per cent NaCl; approximately 1 M NaOH added until pH is 7.0; saline added to make 100 ml.; sterilized by "ultrafine" filtration (Corning U. F.); stored at -20°C. Use 40 ml. per 85 ml. of medium.	
Phosphate buffer:	
Na ₂ HPO ₄ ·12 H ₂ O.....	0.010 M
KH ₂ PO ₄	0.0038 M
Stock solution: 40 ml. 0.10 M Na ₂ HPO ₄ ·12 H ₂ O plus 10 ml. 0.15 M KH ₂ PO ₄ (mixture pH 7.1 by glass electrode measurement); sterilized by U. F. filter; stored at 5°C. Use 12.5 ml. per 85 ml. of medium.	
Sodium thioglycollate.....	0.0016 M
1.50 per cent in distilled H ₂ O; sterilized by U. F. filter; stored in glass-stoppered bottle at 5°C.; made fresh every 2 weeks. Use 1.20 ml. per 85 ml. of medium.	
1 (+) cysteine-HCl.....	0.001 M
0.630 per cent in 0.85 per cent NaCl; sterilized by U. F. filter; stored at -20°C. Use 2.5 ml. per 85 ml. of medium.	
Glutathione.....	0.001 M
1.23 per cent in 0.85 per cent NaCl; sterilized by U. F. filter; stored at -20°C. Use 2.5 ml. per 85 ml. of medium.	
Sodium pyruvate.....	0.001 M
0.60 ml. pyruvic acid plus 80 ml. 0.85 per cent NaCl plus approximately 1 M NaOH to bring to pH 7.0 (about 10 ml. required); made to 100 ml. with 0.85 per cent NaCl; sterilized by U. F. filter; stored in brown bottles at -20°C.; made fresh weekly. Use 1.0 ml. per 85 ml. of medium.	
Vitamin mixture:	
Thiamine-HCl.....	1,000 µg./liter
Niacin.....	1,000 µg./liter
2-calcium pantothenate.....	500 µg./liter
Pyridoxine.....	500 µg./liter
Biotin.....	500 µg./liter

For use as basal medium, see page 370.

per cent nitrogen, and the treponemes extracted from the tissue with gentle rocking for approximately 2 hours at 35°C.³ The extracted treponemes are separated from tissue debris and spermatozoa by filtration through a Corning "medium" fritted disc. The filtrate so obtained is slightly opalescent and on darkfield examination⁴ shows 2 or more organisms per field and an occasional red blood cell, but no appreciable tissue debris. As determined by calibration of the microscope, 2 treponemes per field is equivalent to 10⁷ organisms per ml. When the number exceeds this value an appropriate amount of basal medium is added to dilute the suspension so that it contains 10⁷ treponemes per ml. The percentage of motile organisms is also determined at this time by examining 50 successive treponemes in fields selected at random, and this is recorded as the 0-hour reading. Ordinarily, 90 to 98 per cent of the organisms exhibit active motility. While counts of 100 or more treponemes would yield more precise determinations of the percentage of motile organisms, the time required for such extensive counts would become so long that non-specific loss of motility might occur due to exposure of the organisms to atmospheric conditions other than those designed for optimal survival. This limitation is especially serious in reading a large series of test mixtures as are used in the immobilization tests described below, and has resulted in counting 50 organisms as routine in motility determinations.

Source of Complement.—Pools of about 30 to 40 ml. of serum from 10 to 15 guinea pigs are collected and distributed under aseptic conditions in cotton-plugged, rubber-capped tubes which are stored in solid carbon dioxide. As will be shown in the experimental protocols guinea pig serum in the low concentrations used exerts no effect on the motility of *T. pallidum*.

Collection of Sera.—Tests for immobilizing activity have been performed on five groups of sera: (1) from non-syphilitic, apparently healthy rabbits; (2) from untreated rabbits infected

	Final concentration in test mixture
Choline·HCl.....	500 µg./liter
Inositol.....	500 µg./liter
Biotin.....	10 µg./liter
Folic acid.....	10 µg./liter

A stock solution is prepared in 0.85 per cent NaCl and contains 100 × the concentrations stated; sterilized by U. F. filtration; stored in brown bottles at -20°C. Use 1.0 ml. per 85 ml. of medium.

Sodium bicarbonate.....	0.0072 M
1.26 per cent in distilled H ₂ O; sterilized by U. F. filtration; stored in pyrex glass-stoppered bottles at 5°C.; made fresh biweekly. Use 4.8 ml. per 85 ml. of medium. On admixture of serum and complement additional bicarbonate is contributed, resulting in a final concentration of approximately 0.0085 M, a value required for the maintenance of the system at pH 7.0 at 35°C. under 5 per cent CO ₂ . In runs at 30°C. the level of bicarbonate is raised accordingly.	

Add 0.85 per cent NaCl in distilled H₂O to bring medium to 85 ml.

³ In several of the early experiments, extraction was carried out at 30°C., as noted in the experimental protocols.

⁴ Five thousandths ml. of fluid (measured with a Kahn pipette) is placed under a 22 × 22 mm. coverslip and examined with a calibrated darkfield microscope. The number of treponemes in 25 high power fields is multiplied by a calibration factor of 200,000 to determine the number per milliliter. When accurate quantitation of the number of organisms per milliliter is not required, e.g. in reading the immobilization tests for the percentage of motile organisms, a drop of the fluid treponeme suspension approximating 0.005 ml. may be obtained by the use of a wire-looped inoculating needle.

with *T. pallidum* (Nichols strain) for 3 to 9 months; (3) from members of this department and the Medicine I Clinic of The Johns Hopkins Hospital, whose histories were reliable with regard to the absence of syphilitic infection; (4) from patients with darkfield positive primary or secondary syphilis examined either at the Medicine I Clinic of The Johns Hopkins Hospital or at the Rapid Treatment Center of the Baltimore City Hospitals; and (5) from presumably non-syphilitic patients either with acute febrile diseases or with chronic allergic diseases. Serum, obtained aseptically, is stored at -20°C . in cotton-plugged, rubber-capped tubes. Samples to be tested are heated at 56°C . for 30 minutes just prior to use.

Test Procedure.—One and seven-tenths ml. of the filtered treponeme suspension, 0.2 ml. of the serum to be tested, and 0.1 ml. of guinea pig serum (complement) are pipetted into a 25 X 100 mm. pyrex culture tube. In each experimental series a control tube containing 1.7 ml. of treponeme suspension, 0.1 ml. of complement, and 0.2 ml. of ultrafiltrate of serum¹ is included. It is believed that the latter serves as an appropriate control substitute for whole serum, since the concentration of electrolytes is equivalent to that of whole serum, and since some of these electrolytes, e.g. Mg^{++} and Ca^{++} , may affect the outcome of the test (37). The tubes containing these mixtures are incubated at 35°C . in a Brewer anaerobic jar filled with a gas mixture of 5 per cent carbon dioxide and 95 per cent nitrogen. Percentages of motile organisms are determined as outlined above, at intervals of 4 or 8 hours, depending on the nature of the individual experiment, for a total period of 16 hours.

At the termination of each experiment all mixtures of treponemes, serum, and complement which fail to produce immobilization should be tested for the presence of active complement by addition of sensitized red blood cells, in order to guard against false negative results due to anticomplementary effects.

All manipulations are carried out with strict aseptic precautions. The glassware employed is cleaned with a dichromate-sulfuric acid mixture prior to sterilization. All steps in the procedure which involve exposure of the organisms to conditions other than those outlined for a given experiment, e.g. filtration of the treponeme suspension and reading the tests, are carried out as rapidly as possible in order to obtain optimal survival of the organisms.

Morphological Characteristics of Immobilization of Treponemes by Antibody and Complement.—Unlike treponemes observed in material from human or rabbit syphilitic lesions, those suspended in the fluid medium described above exhibit extremely active movements, which are often so rapid that the entire organism appears to vibrate. Moreover, translational or to-and-fro movements are infrequent, i.e., most of the organisms contract and relax rhythmically in a relatively confined area. In addition, the treponemes bend or twist into circular, S-shaped, or V-shaped forms, but generally resume their characteristic motion along the longitudinal axis within a few seconds.

In the experiments previously reported (36) it was noted that sluggish forms are frequently seen when treponemes are exposed to unfavorable nutrient media. By contrast, when immobilization results from the action of antibody and complement, no intermediate phases of motility, i.e. sluggishly motile forms, have been noted. Since all the organisms seen are either fully active or completely non-motile, their differentiation presents no difficulty. Treponemes immobil-

¹ Ultrafiltrate of serum, prepared by Microbiological Laboratories, Flemington, New Jersey.

ized by antibody and complement suffer no appreciable distortion and may exhibit any of the several shapes described above for motile organisms. These facts, as well as the use of suspensions essentially free from tissue debris, render them easily identifiable. Counts of the total number of organisms have been made repeatedly, but no evidence of lysis has been noted, nor has agglutination been observed.

Since motile and non-motile organisms can readily be identified and differ-

TABLE I

Comparison of the Action of Normal and Syphilitic Rabbit Serum on T. pallidum in Vitro, with and without Complement. Initial (0-Hour) Motility of Treponemes: 98 Per Cent*

Serum pool tested	Final serum dilution	Motility of organisms after incubation at 30°C.			
		Without complement		With complement (1/20)	
		12 hrs.	24 hrs.	12 hrs.	24 hrs.
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Normal.....	1/10	92	94	96	84
Normal.....	1/100	92	94	96	92
Normal.....	1/1000	90	94	96	92
Syphilitic*.....	1/10	96	84	2	2
Syphilitic*.....	1/100	92	96	50	10
Syphilitic*.....	1/1000	96	92	92	84
Serum ultrafiltrate control.....		94	96	92	92

* Syphilis serum pool 3: this pool gave a reagin titre of 24, expressed as the highest serum dilution producing flocculation with Eagle antigen. The Eagle test for reagin in the normal pool was negative.

entiated, and since repeated counting of any one motile treponeme in adjoining fields is unlikely in view of the lack of translational movement, it is possible to make accurate determinations of the total number of organisms, as well as of the percentage of those motile.

EXPERIMENTAL PROTOCOLS AND RESULTS

The Comparative Action of Normal and Syphilis Sera on T. pallidum in Vitro.—

A pool of serum from 10 normal albino rabbits and a pool of serum from 10 rabbits infected with *T. pallidum* (Nichols strain) for approximately 6 months, were tested for immobilizing activity as described above, except that extraction of the treponemes and incubation of the test mixtures were carried out at 30°C.

As shown in Table I, neither serum pool exerted a significant deleterious effect on the motility of *T. pallidum* in the absence of guinea pig complement. How-

ever, in the presence of complement a marked reduction in motility of the organisms was produced by the syphilis serum pool, but not by the normal serum pool. Since this effect was manifested only in the presence of complement, it is highly probable that the component of syphilis serum responsible for the immobilization is an antibody against *T. pallidum*. Subsequent experiments appear to justify this assumption.

TABLE II

Influence of Serum Concentration and Time of Incubation at 35°C. on the Rate of Immobilization of T. pallidum by 3 Different Pools of Serum from Untreated Syphilitic Rabbits. 0-Hour Motility: 90 Per Cent

Serum pool No.	Final serum dilution	Complement added 1/20*	Motility of organisms	
			8 hrs.	16 hrs.
			<i>per cent</i>	<i>per cent</i>
1†	1/10	—	90	78
	1/10	+	12	0
	1/30	+	20	0
	1/90	+	42	2
	1/270	+	60	6
2‡	1/10	—	90	80
	1/10	+	6	0
	1/30	+	22	0
	1/90	+	40	2
	1/270	+	60	8
3‡	1/10	—	90	80
	1/10	+	22	4
	1/30	+	40	10
	1/90	+	50	22
	1/270	+	72	40
Serum ultrafiltrate control.....		—	90	80
Serum ultrafiltrate control.....		+	88	89

* Addition of complement is indicated by +, while absence of complement is denoted by —.

† Rabbit three with Eadie antigen were 2, 4, and 24 for pools 1, 2, and 3 respectively.

The Rate of Immobilization as a Function of Time and Serum Concentration.—

Pools of sera were prepared from each of 3 groups of 10 syphilitic rabbits as follows: pool 1 from rabbits infected for 6 months, pool 2 from rabbits infected for 4 months, and pool 3 from rabbits infected for 3 months. Each pool was tested at final dilutions of 1/10, 1/30, 1/90, and 1/270. Motility observations were made after 8 and 16 hours' incubation at 35°C.

As shown in Table II, no immobilizing effect was manifested by the sera in the absence of complement. This confirmed the data in Table I. Moreover,

in the presence of complement the immobilizing effect increased with time and with serum concentration, and, in general, was manifested rather slowly.

Concentrations of serum above 10 per cent have not been used since in previous experimentation on survival of *T. pallidum in vitro* (36) inactivated normal serum at a final concentration of 40 per cent produced a significant inhibition of survival in the absence of complement. In the present experiments it was found that with occasional normal sera a small degree of immobilization occurred at a final concentration of 20 per cent, but none of the normal sera produced significant immobilization at a concentration of 10 per cent.

For these reasons, a final serum dilution of 1/10 and a minimal time of 16 hours' incubation have been used in the survey experiments recorded in Tables VII to XI. Under these conditions the sensitivity of the test is high, and yet control specimens containing normal serum plus complement, or complement alone, do not show an appreciable decrease in the percentage of motile organisms.

The Rate of Immobilization as a Function of Temperature.—

In this experiment treponemes were extracted from 2 rabbit testes at 30°C. Portions of the filtered treponeme suspension were pipetted into tubes containing serum and complement which had been placed at the designated temperature for the preceding 15 minutes. The rate of immobilization at 25°, 30°, 35°, and 40°C. was determined by reading the percentage of motile organisms at 4, 8, and 16 hours.

It is evident from the results given in Table III that the rate of antibody action increased with temperature, and as a result, the sensitivity of antibody detection at any given time interval also became greater. However, significant immobilization of the control suspension was manifest as early as 4 hours at 40°C., but not at 35°C., or lower. Therefore, an incubation temperature of 35°C. was chosen for subsequent experiments in the survey of human and rabbit sera (Tables VII to XI).

The Role of Complement and the Influence of Its Concentration on the Rate of Immobilization.—

Five ml. portions of a pool of fresh undiluted guinea pig sera were treated as follows: (a) heat-inactivated sample—heated at 56°C. for 50 minutes and then stored in solid carbon dioxide; and (b) decomplexed sample—mixed in the cold with a finely divided, washed specific precipitate consisting of 1.14 mg. of rabbit anti-egg albumin nitrogen and 0.24 mg. of egg albumin nitrogen. This mixture was left at 5°C. for 24 hours with occasional agitation, and then the precipitate was removed by centrifugation. The supernate was sterilized by passage through a Corning "ultrafine" bacterial filter. This procedure was designed to remove complement from the serum by fixation on an antigen-antibody complex (38).

The immobilizing activity of syphilitic rabbit serum (syphilis pool 4) was tested in the usual manner with untreated, with heat-inactivated, and with decomplexed guinea pig serum. In one series of controls, saline was substituted for guinea pig serum. Another set of controls contained ultrafiltrate of serum in place of the syphilitic rabbit serum.

The results shown in Table IV demonstrate that heat inactivation or removal of complement by absorption with a specific precipitate, renders guinea pig

um incapable of immobilizing *T. pallida* in conjunction with syphilitic rabbit um. In view of these findings, the immobilizing activity of fresh guinea pig um has been attributed to its complement content.

TABLE III

Influence of Temperature on the Rate of Immobilization of T. pallidum by Varying Concentrations of Rabbit Syphilis Serum Pool 2 in the Presence of 1/20 Complement. 0-Hour Motility: 95 Per Cent

Temperature of incubation °C.	Final serum dilution	Motility of organisms		
		4 hrs. per cent	8 hrs. per cent	16 hrs. per cent
25	1/10	94	80	30
	1/30	96	90	60
	1/90	96	96	70
	Ultrafiltrate control	94	96	90
30	1/10	80	20	0
	1/30	94	30	0
	1/90	96	50	2
	Ultrafiltrate control	96	94	88
35	1/10	10	0	0
	1/30	40	10	0
	1/90	70	20	0
	Ultrafiltrate control	94	90	92
40	1/10	0		
	1/30	0		
	1/90	0		
	Ultrafiltrate control	50		

TABLE IV

The Identification of Complement as the Component of Guinea Pig Serum Which Immobilizes T. pallidum in Conjunction with Antibody in Syphilis Serum. 0-Hour Motility: 95 Per Cent

Guinea pig serum (1/20 final dilution)	Organisms motile after 8 hrs.' incubation at 35°C.		
	1/10 syphilis serum per cent	1/30 syphilis serum per cent	Ultrafiltrate control per cent
Untreated	6	24	86
Heat inactivated	86	85	86
Decomplemented	84	86	99
None*	90	88	90

* 0.1 ml. (0.15 per cent) substituted for guinea pig serum.

In order to test the effect of varying dilutions of complement on rate of immobilization, 1.0 ml. of treponema suspension was mixed with 0.2 ml. of varying dilutions of syphilitic rabbit serum (syphilis pool 2) and 0.2 ml. of guinea pig serum (undiluted, 1:2, 1:4, and 1:8 dilutions

in saline). Motility readings were made on these mixtures after 4 and 8 hours' incubation at 35°C.

As may be seen in Table V, immobilizing activity was manifested only in the presence of complement and approached maximal intensity with a final guinea pig serum dilution of 1/10 to 1/20. While the difference between 1/20 and

TABLE V

Influence of Complement Concentration on the Rate of Immobilization of T. pallidum at 35°C. by Varying Concentrations of Rabbit Syphilis Serum Pool 2. 0-Hour Motility: 96 Per Cent

Final dilution of complement as guinea pig serum	Final serum dilution	Motility of organisms after incubation at 35°C.	
		4 hrs.	8 hrs.
		<i>per cent</i>	<i>per cent</i>
0	1/10	96	94
	1/50	90	94
	1/250	94	92
	Ultrafiltrate control	94	92
1/80	1/10	64	12
	1/50	94	54
	1/250	96	80
	Ultrafiltrate control	94	92
1/40	1/10	58	2
	1/50	84	18
	1/250	94	70
	Ultrafiltrate control	94	94
1/20	1/10	58	0
	1/50	84	4
	1/250	96	64
	Ultrafiltrate control	96	90
1/10	1/10	56	0
	1/50	78	0
	1/250	92	56
	Ultrafiltrate control	90	92

1/40 complement was hardly significant, it appeared advisable to use a final complement dilution of 1/20 in subsequent experiments in order to provide an adequate excess.

Immobilization as a Criterion for Treponemicidal Activity.—In previous experimentation carried out on the survival of *T. pallidum in vitro* (36), data were accumulated which demonstrated that motile organisms retain their virulence. In the present experiments it became necessary to establish the converse, *i.e.*

that non-motile organisms are dead, in order to prove that the effect of the antibody is treponemicidal.

Total counts and the percentages of motile organisms in 3 mixtures (*a, b, c*) of treponemes, serum, and complement were determined after incubation for 16 hours at 30°C. The total (motile plus non-motile) number of organisms was the same in each mixture, *i.e.*, 5×10^8 per ml. The motility readings were as follows: (*a*) complement plus serum ultrafiltrate—80 per cent; (*b*) complement plus normal serum—78 per cent; and (*c*) complement plus syphilis serum—4 per cent. One-tenth ml. samples of each mixture were injected intracutaneously into 4 sites on the backs of each of 2 normal rabbits. Thus, each rabbit received 12 inoculations, consisting of 4 test sites for each of the 3 mixtures. This procedure provided a comparative test of the 3 samples in a single animal, and therefore eliminated the variation in response of different individuals to inocula of the same size.

TABLE VIa

The Incubation Period and Size of Lesions Following Intracutaneous Inoculation of Rabbits with 5×10^8 T. pallida per Site, as a Function of the Percentage of Motile Organisms. Inocula Obtained from Mixtures of Serum, Complement, and Treponemes after Incubation for 16 Hours at 30°C.

Rabbit No.	Treponemes incubated with	Organisms motile in inoculum	Proportion positive sites*	Incubation period		Average diameter of lesions
				Range	Mean	
		<i>per cent</i>		<i>days</i>	<i>days</i>	<i>mm.</i>
15-92	Complement only	80	4/4	6-7	6.5	13
	Normal serum + complement	76	4/4	6-7	6.5	13
	Syphilis serum + complement	4	3/4	13-14	13.5	3.5
15-93	Complement only	80	4/4	7-9	8	13
	Normal serum + complement	76	4/4	7-9	8	11
	Syphilis serum + complement	4	3/4	19-30	24.5	1.5

* Numerator denotes the number of sites showing darkfield-positive syphilitic lesions after 45 days' observation while denominator represents the total number of sites inoculated.

Significant differences in the incubation period for the development of lesions and in the size of the resultant lesions were observed between the sample containing 4 per cent motile organisms and the 2 samples containing 78 and 80 per cent motile organisms, respectively (Table VI a). Since it has been shown that the incubation period varies with the size of inoculum (39), and since the same total number of organisms (*i.e.* motile plus non-motile) was inoculated into each set of sites, it may be concluded that a significant proportion of the organisms in the mixture containing syphilis serum plus complement was rendered non-infectious and presumably dead, though the data do not suffice for quantitative estimation of the number of treponemes so affected. This result does not, however, furnish strict proof that the immobilized organisms are dead since it is possible that the immobilizing antibody renders the organisms highly suscep-

tible to the defense mechanism of the host. Nevertheless, either mechanism of action would implicate the immobilizing antibody as an important factor in the immune processes in syphilis.

In the second test, which was carried out with another batch of *T. pallidum*, one portion of organisms was exposed to complement plus serum ultrafiltrate (control) and another portion to complement plus syphilis serum. After 16 hours at 35°C., 90 per cent of the organisms in the control sample were motile, but all the organisms in the specimen containing the syphilis serum plus complement appeared to be non-motile. One-tenth ml. of each mixture was inoculated intracutaneously into 4 sites on the backs of each of 3 normal albino rabbits, *i.e.*, a total of 12 sites on 3 animals for the 90 per cent motile sample and 12 sites on 3 different animals for the 0 per cent motile sample. The incubation period was determined by daily observation for the development of lesions.

TABLE VIb

The Incubation Period of Lesions Following Intracutaneous Inoculation of Rabbits with 5×10^8 T. pallida per Site, as a Function of the Percentage of Motile Organisms. Inocula Obtained from Mixtures of Serum, Complement, and Treponemes after Incubation for 16 Hours at 35°C.

Rabbit No.	Treponemes incubated with	Organism motile in inoculum	Proportion of positive sites*	Incubation period	
				Range	Mean
		<i>per cent</i>		<i>days</i>	<i>days</i>
16-40	Complement only	90	4/4	6	6
16-41	Complement only	90	4/4	5-6	5.5
16-42	Complement only	90	4/4	5-6	5.5
16-43	Syphilis serum + complement	0	0/4	—	—
16-44	Syphilis serum + complement	0	0/4	—	—
16-45	Syphilis serum + complement	0	1/4	28	

* Numerator denotes the number of sites showing darkfield-positive syphilitic lesions after 48 days' observation while denominator represents the total number of sites inoculated.

As shown in Table VI b, all sites on the 3 rabbits inoculated with the control mixture (treponemes 90 per cent motile) developed typical darkfield-positive lesions within 6 days. On the other hand, only one of the sites inoculated with treponemes exposed to syphilis serum plus complement (0 per cent motile) developed a darkfield-positive lesion (on the 28th day) and all others were still negative at the time this report was compiled, *i.e.*, 89 days after inoculation. On the basis of dosage response curves published by Magnuson *et al.* (39) it may be estimated that in the 0 per cent motile specimen there were less than 500 viable treponemes in the total inoculum of 500,000 organisms. Thus, the percentage of viable organisms was less than 0.1, a value compatible with the 0 per cent motility observed in a count of 50 treponemes. With the qualifications noted in the preceding experiment, this result indicates that *T. pallida*

immobilized by antibody and complement, are non-infectious and presumably dead. Observations on these test animals continue.

Survey of Individual Rabbit Sera for Immobilizing Antibody.—

Individual serum specimens collected from a group of 20 non-syphilitic, apparently healthy rabbits selected at random from the laboratory stock and from a group of 20 rabbits with

TABLE VII

Survey of Sera from 20 Non-Syphilitic Rabbits for Immobilizing Antibody. All Sera Inactivated at 56°C. for 30 Minutes, and Tested at a Final Dilution of 1/10. 0-Hour Motility: 94 Per Cent

Serum No.	Reagin titre*	Motility of organisms after incubation at 35°C. for 16 hrs.	
		Without complement	With complement
		<i>per cent</i>	<i>per cent</i>
Ultrafiltrate control		—	90
101	0	90	90
107	0	88	88
108	0	90	86
112	0	90	90
113	0	92	70
114	0	90	92
115	0	92	92
116	0	92	90
117	±	90	88
118	0	92	90
119	0	92	92
139	0	92	90
140	±	90	76
141	0	92	92
142	0	84	80
143	0	90	82
144	0	82	84
145	0	86	80
146	0	88	80
147	0	82	70

* Reagin titre expressed as the highest serum dilution producing flocculation with Eagle antigen.

proved syphills of 3 to 6 months' duration, were tested for immobilizing activity and for reagin titre. The tests for immobilizing antibody were carried out at 35°C. with freshly isolated, three-free *T. pallida* in the presence of 1/20 complement. All sera were used at a final dilution of 1/10, and readings were made after 16 hours' incubation.

The results of this survey, shown in Tables VII and VIII, were evaluated in terms of the ratio between the percentages of motile organisms in the tube containing serum alone and the tube containing serum plus complement. A per-

centage difference of less than 10 between these two tubes is not considered significant. If the difference is more than 10 but less than 25, a slight degree of specific immobilization is indicated, and if greater than 25, the test is interpreted as significantly positive. In addition, a control tube containing serum ultrafiltrate and complement was included in each experimental series in order to determine the degree of survival of the organisms in the absence of whole serum.

TABLE VIII

Survey of Sera from 20 Untreated Syphilitic Rabbits for Immobilizing Antibody. All Sera Inactivated at 56°C. for 30 Minutes, and Tested at a Final Dilution of 1/10. 0-Hour Motility: 94 Per Cent

Serum No.	Duration of infection	Reagin titre	Motility of organisms after incubation at 35°C. for 16 hrs.	
			Without complement	With complement
	mos.		per cent	per cent
Ultrafiltrate control	—	—	—	90
103	7	8	80	0
104	9	8	90	0
105	8	2	92	2
106	6	32	90	0
109	8	1	92	0
110	6	8	90	0
111	6	4	90	4
120	6	24	88	0
121	6	8	92	0
122	6	8	82	0
123	6	64	94	0
126	6	8	90	4
130	4	8	80	0
131	4	64	88	0
132	4	12	88	0
133	4	64	92	12
148	4	8	82	0
149	3	32	84	12
150	3	64	90	4
151	3	8	90	0

On the basis of this arbitrary classification, 17 of 20 sera from normal rabbits were negative and 3 showed a slight degree of immobilizing activity. On the other hand, all 20 sera from syphilitic rabbits showed a marked degree of immobilizing activity. With these 20 sera the failure of antibody to produce immobilization in the absence of complement was again verified.

Survey of Individual Human Sera for Immobilizing Antibody.—Since tests for immobilizing activity were negative in the majority of instances with sera from non-syphilitic rabbits, and consistently positive with sera from syphilitic rab-

bits, it became of obvious interest to carry out a preliminary survey of sera obtained from similarly classified human beings.

For this survey the cases for testing were carefully selected. The 20 sera classified as non-syphilitic were obtained from departmental personnel whose histories were reliable with regard to the absence of syphilitic infection. On the other hand, sera classified as syphilitic were obtained from proved cases of syphilis, *i.e.* darkfield-positive primary or secondary cases, with-

TABLE IX

Survey of Sera from 20 Non-Syphilitic Healthy Adult Human Beings. All Sera Inactivated at 56°C. for 30 Minutes, and Tested at a Final Dilution of 1/10 in the Presence of 1/20 Guinea Pig Complement. 0-Hour Motility: 94 Per Cent. All Sera Negative by Eagle Flocculation Test

Serum No.	Motility of organisms after incubation for 16 hrs. at 35°C.
	<i>per cent</i>
Ultrafiltrate control	92
7	90
9	88
10	90
11	92
12	90
13	92
15	90
16	92
17	94
18	92
31	88
61	80
62	90
63	88
64	90
65	92
66	90
67	88
68	80
(9)	90

out regard to the state of the standard serological test. Cases of latent syphilis have been avoided to date since frequently this diagnosis is necessarily made on the basis of standard serological tests, which may prove to be less definitive in this stage of the disease than the test here described. The technique for testing the human sera was identical with that used in testing the rabbit sera.

In general the results obtained with human sera (Tables IX to XI) correlate with those obtained with the rabbit sera (Tables VII and VIII). None of 20 normal sera showed significant immobilizing activity. Of 20 sera from cases of primary syphilis, 7 were negative, 10 were positive, and 3 showed a slight degree

of immobilizing activity. All 20 sera from cases of secondary syphilis, produced a marked immobilizing effect indicating the presence of significant antibody serum levels. In addition, 20 sera from patients with diseases other than syphilis were tested. Sixteen showed no immobilizing activity but the other 4 yielded inconclusive results since immobilization of the treponemes occurred both in

TABLE Xa

Survey of Sera from 20 Human Cases of Darkfield-Positive Primary Syphilis for the Presence of Immobilizing Antibody. All Sera Inactivated at 56°C. for 30 Minutes, and Tested at a Final Dilution of 1/10. 0-Hour Motility: 94 Per Cent

Serum No.	Reagin titre	Motility of organisms after incubation for 16 hrs. at 35°C.	
		Without complement	With complement
		<i>per cent</i>	<i>per cent</i>
Ultrafiltrate control	—	90	88
28	32	94	72
33	32	88	0
35	8	88	88
41	16	92	64
70	1	90	72
71	1	86	80
72	1	88	70
86	16	82	4
89	24	92	10
92	0	86	88
93	0	88	90
94	16	82	0
95	32	84	18
102	0	86	88
115	8	88	62
116	0	90	86
122	4	80	80
123	0	86	50
134	96	88	22
135	32	84	44

the complement-free control tube and in the test sample containing complement. Since these 4 sera were obtained from patients receiving penicillin therapy it is possible that their sera contained sufficient amounts of this antibiotic to produce the immobilization observed in both tubes.

The Relationship of Immobilizing Antibody to Reagin.—In order to study the relationship of reagin to immobilizing activity of syphilis serum, the following absorption experiments were carried out.

A primary dilution of Eagle antigen was prepared by mixing 1 volume of an alcoholic solution of standard antigen with 2 volumes of 0.85 per cent saline. After this mixture had aged

at 5°C. for 24 hours, the lipoidal particles were sedimented by centrifugation at 20,000 R.P.M., and then brought to original volume with 0.85 per cent saline. The lipoidal particles were then sedimented again from 2 ml. and 5 ml. portions for the first and second absorptions, described below.

First Absorption.—Ten ml. of rabbit syphilis serum pool 4 was mixed with the washed sedimented lipid from 2 ml. of Eagle antigen emulsion, and the mixture thoroughly agitated for

TABLE X b

Survey of Sera from 20 Human Cases of Darkfield-Positive Secondary Syphilis for Presence of Immobilizing Antibody. All Sera Inactivated at 56°C. for 30 Minutes, and Tested at a Final Dilution of 1/10. 0-Hour Motility: 92 Per Cent

Serum No.	Reagin titre	Motility of organisms after incubation at 35°C. for 16 hrs.	
		Without complement	With complement
		<i>per cent</i>	<i>per cent</i>
Ultrafiltrate control	—	92	88
19	64	90	62
22	64	88	0
32	64	92	4
34	128	88	16
36	32	88	4
37	48	92	8
38	64	88	20
42	16	80	0
43	24	92	0
54	24	90	4
77	64	94	0
78	24	84	20
79	64	92	6
97	6	86	6
99	128	84	0
117	128	86	0
119	64	80	0
136	12	82	0
138	96	78	0
142	64	78	0

10 minutes on a Kahn shaker. The resultant flocculate was separated from the serum by centrifugation at 20,000 R.P.M. for 30 minutes. The supernate (once absorbed serum) was pipetted off and filtered through a Corning "ultrafine" bacterial filter to remove any unsedimented flocules, as well as to sterilize the serum.

Second Absorption.—Five ml. of the filtrate obtained above (once absorbed serum) was mixed with the washed sedimented lipid from 5 ml. of Eagle antigen emulsion, and treated exactly as in the first absorption.

As shown in Table XII, one absorption of the syphilis serum with Eagle antigen reduced the reagin titre from 16 to 2, while a second absorption removed all

titratable reagin activity. However, no detectable decrease in immobilizing activity resulted from this treatment when the sera were tested in the usual

TABLE XI

Survey of Sera from Patients with Diseases Other Than Syphilis for the Presence of Immobilizing Antibody. All Sera Inactivated at 56°C. for 30 Minutes, and Tested at a Final Dilution of 1/10. 0-Hour Motility: 90 Per Cent*

Serum No.	Type of disease	Duration of infection	Motility of organisms after 16 hrs. at 35°C.	
			Without complement	With complement
			per cent	per cent
Ultrafiltrate control	—	—	—	88
108	Acute streptococcal laryngotracheobronchitis	5 days	88	84
110	<i>H. influenzae</i> meningitis	9 days	90	86
114	Infectious mononucleosis	8 days	82	80
149	Eczematoid dermatitis	3 mos.	74	86
150	Atopic dermatitis	10 yrs.	76	84
151	Seborrheic dermatitis	3 mos.	70	82
306	Acute tonsillitis	2 days	80	84
307	Mumps	2 days	88	80
308	Measles	3 days	84	80
309	Cellulitis	7 days	84	78
313	Diphtheria	1 day	82	82
314	Polioomyelitis	7 days	96	90
316	Atypical pneumonia	14 days	88	80
318	Brucellosis	2 yrs.	84	86
319	Tuberculous meningitis	5 mos.	90	80
320	Pertussis	14 days	78	88
109	Lymphocytic meningitis (questionable etiology)†	4 days	20	16
111	Pneumococcal meningitis‡	3 days	16	2
112	Diphtheria‡	16 days	14	12
113	Pneumonia‡	15 days	20	18

* All sera from this group of patients were negative for reagin with the Eagle and cardiolipin flocculation tests.

† These patients were receiving penicillin therapy at the time serum specimens were obtained. Inconclusive results were obtained since non-specific immobilization occurred in the absence of complement.

manner. Repetition of this experiment with another rabbit serum pool (syphilis serum pool 3) yielded similarly clear-cut results.

Therefore it may be concluded that the immobilizing and reagin activities are due to separate antibodies.

TABLE XII

*Titration of Immobilizing Activity of Rabbit Syphilis Serum Pool 4 before and after
Absorption of Reagin by Flocculation with Eagle Antigen. Complement
Dilution—1/20. 0-Hour Motility: 92 Per Cent*

No. of absorptions with Eagle antigen	Reagin titre	Final serum dilution	Motility of organisms after 8 hrs. at 35°C.
0	16	1/10	0
		1/50	0
		1/250	18
		1/1250	48
1	2	1/10	0
		1/50	2
		1/250	16
		1/1250	50
2	0	1/10	0
		1/50	0
		1/250	14
		1/1250	48

TABLE XIII

*The Relative Heat Stability of Reagin and Immobilizing Antibody. Immobilization Test
Carried Out with Rabbit Syphilis Serum Pool 2, with 1/20 Complement. 0-Hour
Motility: 94 Per Cent*

Serum heated	Reagin titre	Final serum dilution	Motility of organisms after incubation at 35°C.	
			8 hrs.	16 hrs.
			<i>per cent</i>	<i>per cent</i>
Ultrafiltrate control	—	—	88	90
Unheated control	—	1/10	14	2
		1/30	20	10
		1/90	50	14
56°C. 30 min.	4	1/10	10	2
		1/30	30	18
		1/90	54	18
60°C. 30 min.	3	1/10	12	6
		1/30	30	12
		1/90	54	18
64°C. 30 min.	1	1/10	10	2
		1/30	30	14
		1/90	54	14
68°C. 30 min.	0	1/10	14	2
		1/30	46	12
		1/90	60	16

The Relative Thermostability of Reagin and Immobilizing Antibody.—

Five samples of 3 ml. each from rabbit syphilis serum pool 2, were heated for 30 minutes at temperatures of 56°, 60°, 64°, and 68°C., respectively. A standard Eagle flocculation test was performed on each of the samples, and tests for immobilizing antibody were carried out with final serum dilutions of 1/10, 1/30, and 1/90. Motility readings were made at 8 and 12 hours.

The results shown in Table XIII demonstrate a progressive drop in reagin titre with increased temperature of heating, but no corresponding decrease in immobilization activity. In order to verify this striking difference in heat sensitivity this experiment was repeated with another sample of the same serum pool. Further to evaluate the stability of the immobilizing antibody at higher temperatures, 3 additional portions were heated at 70°, 72°, and 76°C. for 15 minutes. No decrease in immobilizing activity was noted with the samples heated at 70° and 72°C. The portion heated at 76°C. could not be tested because the serum proteins had coagulated.

Although the ability to withstand heating at 72°C. for 15 minutes appears unusual for an antibody, similar heat stability has been noted in other studies. For example, Jones (40) found that rabbit agglutinin against the flagella component of the hog cholera bacillus withstood heating at 70°C. for 20 minutes and was not completely destroyed in 20 minutes even at 90°C. Other examples are cited by Marrack (41).

Further studies on the physicochemical characterization of the immobilizing antibody are planned.

DISCUSSION

Although the number of sera tested in the present study is comparatively small, the results clearly show that sera from syphilitic rabbits or human beings with infection beyond the primary stage usually exert a marked immobilizing action on virulent *T. pallidum in vitro*, while this activity is virtually absent from normal sera. Like bactericidal and hemolytic phenomena, this immobilization effect occurs only in the presence of complement and therefore appears to be due to the action of specific antibody.

The immobilization of treponemes by antibody and complement is a relatively slow process, as contrasted to other specific *in vitro* antibody activities. As shown in Tables II and III, the speed of immobilization increases with the concentration of serum and with the temperature of incubation, but even with conditions designed for maximal velocity (1/10 serum dilution and 35°C. incubation) several hours are required for significant degrees of immobilization to occur (Table III). Concentrations of serum higher than 10 per cent or temperatures above 35°C. have not been employed since under these conditions non-specific immobilization of treponemes may occur in control suspensions.

The inability of previous investigators to obtain evidence of immobilization

of *T. pallidum* by immune serum may be attributed primarily to the use of poor experimental conditions resulting in the non-specific loss of motility of treponemes before the effect of antibody and complement could become manifest. This difficulty has been overcome in the present investigation by the use of specially designed procedures and media which permit the maintenance of *T. pallidum in vitro* in a highly active state for several days. Failures to obtain prolonged survival of control treponemes have been encountered on rare occasions in the present experiments, but it appears likely that these failures are due either to the deterioration of some labile constituent of the basal medium or to unknown factors inherent in testicular lesions which inadvertently have been allowed to progress beyond the stage of early syphilitic orchitis before removal.

Following the determination of conditions which appeared optimal for the detection of immobilizing antibody, a preliminary survey of animal and human sera was undertaken. While the antibody titre can be estimated with a fair degree of precision in terms of an end-point based on 50 per cent immobilization at a given time, limitations of time and material precluded such quantitation in these preliminary experiments. The surveys were therefore carried out under conditions of optimal sensitivity, *i.e.*, with a final serum dilution of 1/10 and incubation at 35°C. for 16 hours. The 16 hour incubation interval was selected so as to provide a sufficiently long period for low titre sera to exert a significant immobilizing effect, as well as to be convenient from the standpoint of carrying out the necessary laboratory procedures.

Of 20 cases of human primary syphilis tested (Table X *a*), 10 showed a marked immobilizing action, 3 exhibited weak activity, and 7 were without effect. In addition, a marked degree of immobilizing activity was exhibited by all the sera from 20 cases of secondary syphilis (Table X *b*). Since the infection had been present for several weeks longer in the secondary than in the primary cases, it is probable that the antibody level in a given individual increases with the duration of the disease. However, as yet no cases of late syphilis in human beings have been studied to verify this conclusion.

All the 20 sera from normal human beings examined (Table IX) were completely devoid of immobilizing activity, as were 16 of the 20 sera from patients with diseases other than syphilis (Table XI). The other 4 yielded inconclusive results since immobilization occurred both in the complement-free control tube and in the test mixture containing complement. These 4 patients were receiving penicillin therapy at the time the serum specimens were collected and the non-specific immobilization exerted by their sera was probably due to the penicillin present in the serum (36). Therefore, it appears advisable to collect sera prior to the administration of antibiotic or chemotherapeutic agents to the patient. Otherwise the risk of obtaining such inconclusive results is present, since at least 3 antibiotics, bacitracin (42), penicillin (36), and aureomycin (43), have been shown to immobilize *T. pallidum in vitro*, and would be present in the

sera of patients treated with these agents. However, such "non-specific" immobilization may readily be differentiated from the specific effect due to antibody, since the latter immobilizes treponemes *only* in the presence of complement.

Comparison of sera from 20 syphilitic rabbits infected for periods ranging from 3 to 9 months (Table VIII) with sera from 20 normal rabbits (Table VII) yielded similarly striking results. All the sera from syphilitic animals gave strongly positive reactions, while 17 of the 20 normal sera were completely negative. The other 3 normal sera exhibited low degrees of immobilizing activity. However, the presence of traces of antibody in the serum of an occasional rabbit might be expected in view of its possible infection at one time with *T. cuniculi* (rabbit venereal spirochetosis), an organism immunologically related to *T. pallidum* (44).

It appeared of prime importance to examine the relation of the immobilizing antibody to reagin. From the data presented in Tables II and VII to X, no direct correlation is apparent, except that both activities are more strongly manifest in secondary than in primary syphilis in human beings (Table X *a* and X *b*). The lack of relationship is shown clearly in the absorption experiment outlined in Table XII, which indicates that the immobilizing and reagin activities are due to separate antibodies. Moreover, since it is well known that the level of reagin is relatively low in late syphilis, when resistance to reinfection is high (26, 27), it apparently plays no rôle in immunity. On the other hand, the immobilizing antibody appears to kill *T. pallidum* (Tables VI *a* and VI *b*), and therefore its appearance may be associated with, and possibly responsible for, the development of the immune state. It may also follow that the immobilizing antibody is identical with the antibody demonstrated in human sera by means of passive protection tests in rabbits (6-10).

As an *in vitro* technique for the detection and measurement of specific antibody produced during syphilitic infection, the present immobilization test offers a convenient approach to the study of certain fundamental problems in the biology of the disease in animals and man. These include a study of: (*a*) the incidence and titre of immobilizing antibody in various stages of syphilitic infection in human beings; (*b*) the rate of appearance of immobilizing antibody during experimentally induced syphilis in rabbits, and its quantitative relationship to the development of immunity, as measured by resistance to reinfection; (*c*) the possible absence of immobilizing antibody in non-syphilitic individuals whose sera contain reagin, *i.e.*, the "biologic false positive" reactors; (*d*) the immunological relationships among experimentally induced *T. pertenuis* (yaws), *T. cuniculi* (rabbit venereal spirochetosis), and *T. pallida* (syphilis) infections in rabbits; and (*e*) the possible immunological variations among different strains of *T. pallidum*.

In addition, it will be necessary to investigate thoroughly the various tech-

nical and theoretical aspects of the immobilization test before it can be applied to clinical diagnostic problems. For example, some diseases other than syphilis may produce antibody which cross-reacts with and immobilizes *T. pallidum*. If, however, the occurrence of such cross-reactions is rare the immobilization test may be of value in the study of sera from patients with so called "biologic false positive" reactions, *i.e.*, individuals whose sera contain reagin despite the absence of syphilitic infection. The perplexing problem encountered with this group of individuals involves the differential diagnosis of a "biologic false positive" reaction from the positive test for reagin found in patients with latent syphilis in whom no evidence of the disease is apparent on physical examination. Since reagin and immobilizing activities appear to be due to separate antibodies, it is possible that the stimuli which incite the production of reagin in a non-syphilitic individual may not give rise to immobilizing antibody. Moreover, in view of its probable association with active immunity, it may be anticipated that the titre of immobilizing antibody, unlike reagin, will remain relatively high in untreated syphilitics whose infection progresses to the latent phase. If this assumption proves correct in subsequent studies, the detection of immobilizing antibody in the sera of patients with latent syphilis should present no difficulty, particularly since the present preliminary studies indicate that the immobilization test as outlined is sufficiently sensitive to detect antibody in a fair number of sera from cases of primary syphilis in which comparatively low levels would be expected to be present. On this basis, the persistent absence of immobilizing antibody would be of aid in excluding the diagnosis of syphilis.

In addition, further studies are in progress in an attempt to define more clearly the factors which influence quantitation and reproducibility of the immobilization test, as well as to simplify the rather complex bacteriological procedures employed at present, so that the test may be applied more readily on a large scale in studies on the immunology of syphilis.

SUMMARY

Treponema pallidum were extracted from rabbit testicular syphilomas and suspended in a special medium in which the organisms remain motile and infectious for several days. On incubation of such suspensions with syphilitic rabbit or human sera and guinea pig complement, the treponemes became non-motile and lost their capacity to infect rabbits. Various factors affecting this immobilization have been investigated.

In a preliminary survey of individual sera, immobilizing antibody could be detected in the majority of sera from syphilitic animals and human beings, but was absent in almost all the normal sera examined.

It could be demonstrated that the immobilizing and reagin activities of syphilitic sera are due to separate antibodies.

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STUDIES ON ACUTE DISSEMINATED ENCEPHALOMYELITIS PRODUCED EXPERIMENTALLY IN RHESUS MONKEYS

IV. DISSEMINATED ENCEPHALOMYELITIS PRODUCED IN MONKEYS WITH THEIR OWN BRAIN TISSUE*

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PLATE 14

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The rapid production in several laboratories (1-7) of acute disseminated encephalomyelitis in several species by the injection of homologous brain tissue in an emulsion with paraffin oil, aquaphor, and killed tubercle bacilli (for earlier studies without these adjuvants *cf.* references 8 and 9), an immunization procedure introduced by Freund and McDermott (10), has focussed attention on the possibility of autosensitization or autoantibody formation as a mechanism in the causation of certain demyelinating diseases. Since it was possible that individual differences existed among the brain antigens of individuals of a given species and therefore that injection of brain emulsions from one individual into another might be more or less comparable to the injection of a foreign antigen, it was considered of importance to establish whether or not encephalomyelitis could be produced in an animal by the injection of an emulsion of its own brain tissue. Accordingly, *rhesus* monkeys from which the right frontal lobe had been excised were given injections of emulsions with adjuvants prepared from their own individual brain tissue. These animals developed typical acute disseminated encephalomyelitis.

EXPERIMENTAL

Right frontal lobectomies were performed on six *rhesus* monkeys. The brain tissue removed, from 3.3 to 5.5 gm. per animal, was kept frozen in solid carbon dioxide until the animals had recovered. Emulsions of the brain tissue with aquaphor and paraffin oil containing heat-killed tubercle bacilli were prepared as previously described (2). Each animal received three 1 ml. intramuscular injections, a week apart, of the emulsion prepared from its own brain tissue. Animals were carefully observed for symptoms which could not be attributed to the effects of the surgery and were sacrificed and necropsied when their condition became such that they appeared unlikely to survive another day.

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RESULTS

Five of the six animals developed symptoms in from 4 to 28 days following the third inoculation. These included ptosis of an eyelid, dilatation of the

TABLE I

Production of Acute Disseminated Encephalomyelitis in Lobectomized Rhesus Monkeys Injected with Their Own Brain Tissue Plus Adjuvants

Monkey No.	Days signs first noted after 1st injection	Signs observed	Day of death	Necropsy findings*
2-86		None; found dead	19	Negative for encephalomyelitis; acute, suppurative cerebrospinal leptomenigitis and ventriculitis complicating cranial operation
2-87	41	Ptosis, left eyelid; blindness, dilated pupils	52	Positive; subacute and chronic perivascular and coalescent, demyelinating lesions found chiefly in optic nerves and sparsely in rest of brain
2-88	26	Trunk ataxia, intention tremor	26(S)	Positive; acute and subacute perivascular and coalescent, inflammatory and demyelinating lesions in brain most severe in brain stem and cerebellum
2-89	20	Dilated pupils; blindness, convulsive seizures	23(S)	Positive lesions as in 2-88; most marked in optic nerves and cerebrum and less intense in brain stem and cerebellum; sparse in spinal cord†
2-98	18	Trunk ataxia; generalized tremors	19(S)	Positive; few acute and many subacute lesions as in preceding animals most marked in pons, and less extensive in cerebellum and cerebrum
3-00	23	Dilatation of pupils, generalized tremors	23(S)	Positive; acute and subacute lesions as in above animals of considerable severity in the brain stem and optic nerves, less marked in the cerebrum and least intense in the cerebellum; sparse lesions in the spinal cord

Animals received three 1.0 ml. intramuscular injections at weekly intervals.

S = sacrificed.

* All animals showed scars of right frontal lobectomy and secondary degeneration consequent to it.

† Right frontal lobectomy followed by local abscess formation, aspirated, instilled with penicillin, subsided before brain inoculation; local granulation tissue.

pupils, reduced vision or blindness, intention tremor, ataxia, and fine generalized tremors (Table I). One monkey in which complete blindness, dilatation of the pupils, and ptosis of the left eyelid were the only symptoms, showed numerous

lesions in the optic nerves (Fig. 1) and rare limited lesions in the cerebral white matter. In this animal death occurred 53 days after the first inoculation, 39 days after the last, and 11 days after the first symptoms. Inflammation was absent in the optic nerve lesions and absent or slight and limited to perivascular lymphocytes in the others. In the other four animals lesions were found in the cerebrum, cerebellum, brain stem, and optic nerves (Fig. 2) and in two in the spinal cord as well. These had died in from 5 to 19 days after the last inoculation and 1 to 3 days following the first symptom. The lesions were acute or subacute (Fig. 3), perivascular (Figs. 3 and 4), and frequently coalescent. Their characteristics were in every respect like those previously described in acute disseminated encephalomyelitis in the monkey (2, 11).

In the sixth monkey, found dead 6 days after the last inoculation, an acute suppurative cerebrospinal leptomeningitis and ventriculitis was encountered. In this animal no lesions were found in the brain or spinal cord and the acute leptomeningitis was probably a complication of the operation although no infection of the scalp wound was discovered and death occurred 29 days after the operation. There were tuberculous lesions in the lungs and liver.

DISCUSSION

The successful production of acute disseminated encephalomyelitis in *rhesus* monkeys by the injection of a portion of their own individual brains which were previously removed surgically and incorporated with adjuvants, provides additional evidence for the hypothesis previously advanced that such pathological changes may be a result of sensitization or antibody formation to an individual's own brain tissue. It should be emphasized, however, that the procedure used for producing the encephalomyelitis is a highly artificial one, and the manner in which such a sequence of events could be initiated in instances of spontaneously occurring encephalomyelitides or in other related diseases is as yet completely unknown.

SUMMARY

Acute disseminated encephalomyelitis has been produced in *rhesus* monkeys by injection of their own brain tissue, removed surgically and incorporated with adjuvants.

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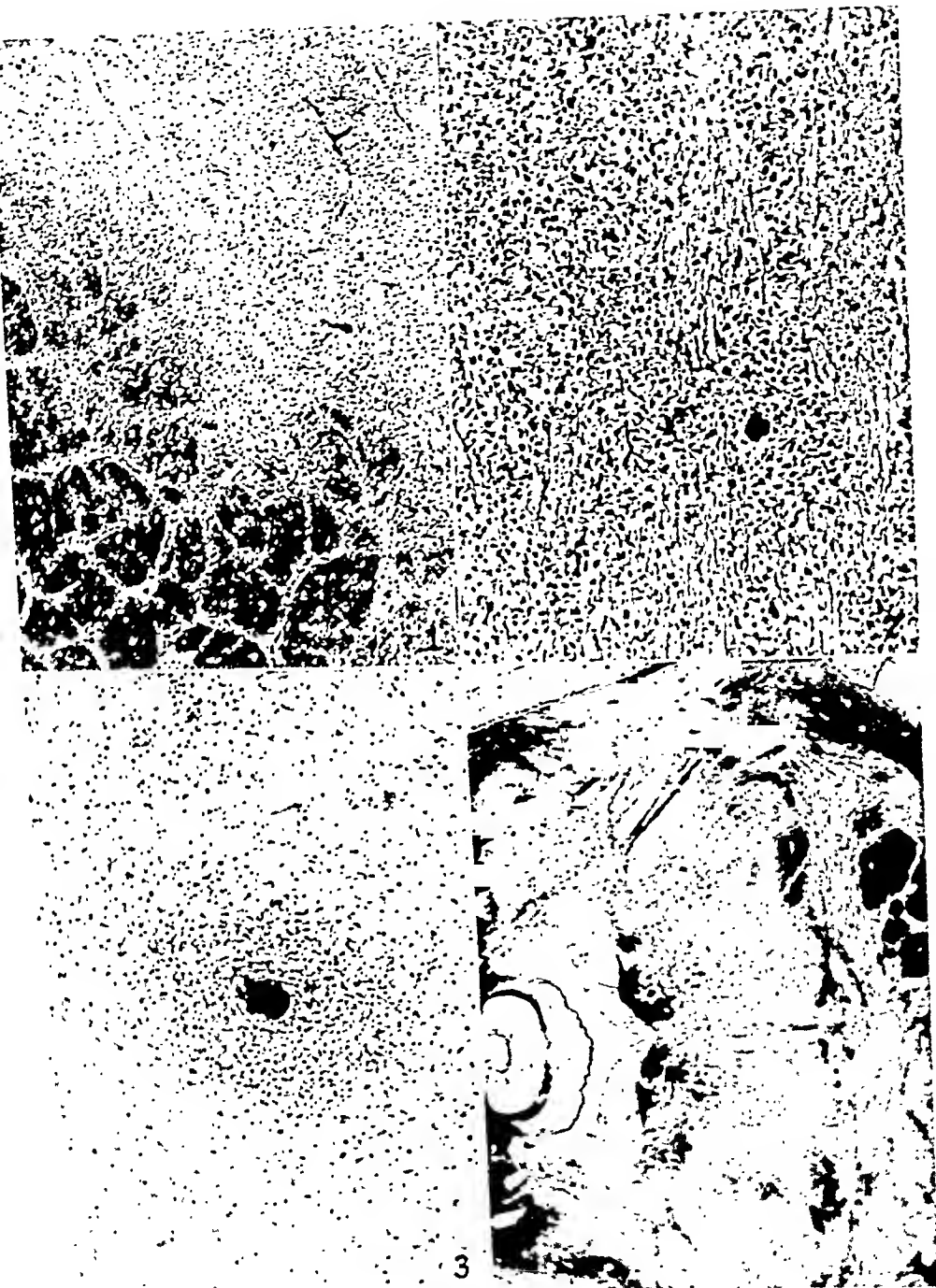
EXPLANATION OF PLATE 14

FIG. 1. Monkey 2-87. Coalescent area of demyelination in optic nerve. Mahon stain. $\times 56$.

FIG. 2. Monkey 2-89. Partial preservation of axones in demyelinated area in optic nerve. Bodian stain. $\times 85$.

FIG. 3. Monkey 3-00. Focal lesion in thalamus. Perivascular infiltration by lymphocytes and associated microglial proliferation. Hematoxylin-eosin stain. $\times 56$.

FIG. 4. Monkey 2-98. Numerous perivascular foci of demyelination in pons. Mahon stain. $\times 4.5$.



ADDITIVE EFFECTS OF CERTAIN TRANSFORMING AGENTS FROM SOME VARIANTS OF PNEUMOCOCCUS

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PLATE 15

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There has been little reason to believe that the bacterial cell contains a special genetic substance or structure, differentiated to perform genetic functions, for it can be imagined that each part of the bacterium reproduces itself in fission, and is thus itself responsible for its own genetic continuity. There are numerous examples of differentiated extranuclear structures, in nucleated cells, which are capable of a high degree of genetic autonomy, and indeed which may be said to reproduce themselves provided a favorable intracellular environment is present. For example, there are the plastids of plants and the centrosomes of animal cells. The genetic autonomy of these structures is apparently compatible with the perpetuation of the cell in constant form. In view of the fact that even in highly differentiated organisms, non-nuclear genetic elements possessing genetic continuity are found, a number of hypotheses can be proposed to explain the constant properties of bacterial clones. No definitive evidence exists as yet to indicate whether or not this constancy may be attributed to the functioning of a nuclear apparatus.

At present one type of evidence suggests that certain inherited traits in bacteria are controlled by localized units. X-rays produce permanent alterations in bacterial cells, as well as in the germ cells of higher organisms. In the latter case, the site of action of the x-rays has been localized in the nuclear genes. Although quantitative data on the production of heritable changes in bacteria suggest that here too the x-rays are acting upon certain localized structures, and not upon structures distributed generally throughout the cell, rigorous proof of the "target theory" is lacking in bacteria (1). Hence, the radiation studies are only presumptive evidence that perpetuation of the affected characters is achieved through the activities of single, localized determinants within the bacteria studied.

From an entirely different approach, evidence has been obtained that the production of at least one specific complex element of a bacterium is determined by a chemically unrelated cellular component. Thus, it appears that production of the pneumococcal type-specific capsular antigen is dependent upon the presence of a specific deoxyribonucleic acid within the bacterium. Bacteria which do not form the specific antigen may interact with isolated specific

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nucleic acid and, as a result of this interaction, their progeny continue to produce not only the specific antigen, but also more of the specific nucleic acid used to induce the change (2-4).

As the transformed organism divides, its progeny are found to form both a capsule, and the transforming principle, just as do encapsulated pneumococci of natural origin. It appears justified, therefore, to visualize the transforming principle much as the geneticist pictures genes, as a self-duplicating agent which initiates a series of reactions, involving in the case of the transforming principle probably both the synthesis of a specific enzyme and a capsular polysaccharide.

In the past, colonial morphology and immunological characteristics have served as criteria for differentiating pneumococcal cultures. As a consequence of the transformation studies, however, characterization of smooth and rough races of pneumococci may be made by yet another criterion: the gene-like transforming principle found in each of the smooth strains thus far studied, is apparently absent in the non-encapsulated or rough races (3, 4). While races of pneumococci have been analyzed with respect to their antigenic structure, no extensive study of the group has been made considering as an essential differential characteristic the presence or absence of specific transforming principles. The present study is essentially an inquiry into the differences between several races of pneumococci, as manifest in the transforming activities of desoxyribonucleates obtained from these races.

The present report will cover the following points:

1. From the R strain used customarily in transformation experiments, a permanently altered strain, ER, was obtained which forms an extremely rough colony on solid media. Evidence will be presented which indicates that strain ER differs from R in one essential feature. R pneumococci possess a transforming principle capable of transforming ER pneumococci into R, while ER pneumococci lack such a principle. SIII pneumococci possess not only the transforming principle active in transforming R pneumococci into SIII, but also the new transforming principle active in changing ER pneumococci into R.

2. Two of a series of mutated forms of Type III smooth pneumococci have been studied. The experiments to be discussed indicate that the altered condition of these strains is attributable to mutation of the transforming principle found uniquely in SIII pneumococci. Experiments upon an analogous strain of Type II pneumococcus have recently been described (5). The more extensive data to be discussed in connection with the present experiments upon the SIII variant strains provide strong evidence against the suggestion of these authors that the transformation of an R pneumococcus into an S requires the acquisition of more than one "character" by the pneumococcus undergoing transformation. Neither the material published by these authors, nor the present experiments, give information as to whether the transformation of R into S involves the action of one or more active particles of the desoxyribonu-

cleate fraction of the S cell. However, the present experiments exclude the possibility that the mutated SIII races are races in which a normally multipartite SIII transforming agent is only partially present. Phrased in the terms of genetics, the present experiments suggest that the mutated and normal SIII transforming principles are related to each other as are the genes of an allelic series, and not as genes of a polymeric series of which more than one must be present for normal antigen production.

3. Two kinds of two-step transformations were found possible. From the differences between these two-step transformations it is concluded that in certain cases the transformation phenomenon consists of an interaction or exchange occurring between a transforming principle within the pneumococcus and a similar principle in the environment.

Materials and Methods

To obtain transformation *in vitro* it is necessary to provide special environmental conditions for the susceptible pneumococcus. It has been shown that the essential components of this environment, in addition to nutrient materials, are (1) an agglutinating agent and (2) an accessory protein constituent of serum, which, when present in whole serum, can be inactivated by dialysis (6). Except for certain modifications which will be discussed below, essentially identical techniques have been employed for all the various transformations to be described, and in every case the above environmental conditions had to be met.

Agglutinating Agents

In transforming an R strain, either a whole serum was employed which contained enough R agglutinins to provide completely sedimented growth of the R inoculum, or the agglutinins were added in the form of a concentrated gamma globulin fraction of the serum of rabbits immunized with rough pneumococci. A crude fractionation of the serum was made by dialyzing the serum against 18 per cent sodium sulfate. The resultant precipitate was collected by centrifugation and washed by resuspension in 18 per cent sodium sulfate. The washed precipitate was dissolved in a small volume of distilled water and this solution dialyzed against physiological saline. Sterility was maintained throughout. Following dialysis, the agglutination activity of the sterile solution was titrated by growing R pneumococci in medium containing varying amounts of the antibody solution. This kind of titration is called a "thread test" and will be referred to by this name at several points in the following report. Most preparations of R agglutinins contained enough antibody to give completely sedimented growth of the R bacteria at a concentration of 1 part of the agglutinin solution to 1000 parts of broth. Consequently, this was the concentration usually employed for transformation experiments.

In some instances SIII organisms were transformed, and to agglutinate these pneumococci a more delicate system was substituted. Two techniques were employed. The solutions of *Escherichia coli* could be employed to agglutinate the SIII bacteria provided the specific polysaccharide formed by these pneumococci was constantly removed from the cell surface during growth. This could be done by using the enzyme which specifically hydrolyzes the Type III polysaccharide (7), since the enzyme in no way interferes with the transformation process. An alternative to this is, Type III antiserum could be employed to agglutinate the SIII organisms either in the form of whole rabbit serum, or in the form of a twice precipitated

gamma globulin fraction of Type III antipneumococcus horse serum. The concentrations used were sufficient to give agglutinated growth of the inoculum of SIII cells. In the case of the globulin fraction of horse serum, 1 part of the solution was added to 1000 parts of broth.

Sources of Accessory Serum Factor

The second serum constituent, which is distinct from the agglutinins, was added either as whole human pleural fluid, of known activity in supporting transformations, at concentrations of 7 to 10 per cent, or in the form of a purified fraction of bovine serum. In a future publication a report will be made on the experiments performed in collaboration with Dr. O. T. Avery and Dr. R. D. Hotchkiss, which have led to the use of bovine serum albumin in place of whole serum in effecting transformations. As routine a 4 per cent solution of fraction V of bovine serum albumin, prepared by Armour and Company according to the techniques developed in the laboratory of Dr. E. J. Cohn, was adjusted to a pH of 7.0 and filtered through Coors No. 3 porcelain filters for sterilization. This solution was added to broth in amounts ranging from 3 to 5 parts per 100, this range of concentrations having been found to be in excess of the minimum necessary to obtain regular transformations with active transforming extracts. Sodium pyrophosphate added to the transforming environment was found to eliminate some variability in the results, and consequently was added to the albumin solution just prior to its use in an experiment. 1 part of a 1/15 solution of sodium pyrophosphate was mixed with 10 parts of the 4 per cent albumin solution.

Preparation of Transforming Principles

The improved method of isolating transforming principles from pneumococci was used (4). When only a few tests of biological activity of a given preparation were to be performed, large scale preparations were not made, and hence extensive purifications could not be carried out. The preparations of transforming principles made on a small scale contained several milligrams of desoxyribonucleate, and in addition contained ribonucleic acid, somatic polysaccharide, and in some cases, capsular polysaccharide. All transformations employing the normal SIII transforming principle, and all employing the desoxyribonucleate of R cells, were performed with highly purified preparations of desoxyribonucleic acid. The majority of transformations with the transforming principle referred to as SIII-1 were also done with highly purified desoxyribonucleate. The remaining preparations were made as described below.

The strain from which a transforming extract was to be prepared was grown from a single colony isolation, and a heavy inoculum seeded into 1500 cc. of beef heart infusion-neopeptone broth. After 17 hours' incubation at 37°C. the cells were harvested by centrifugation. Following resuspension in 10 cc. of 0.1 M sodium citrate-0.1 M saline solution, 0.05 cc. of a 10 per cent solution of sodium desoxycholate was added to induce lysis. Lysis usually occurred within 15 minutes at room temperature. The highly viscous lysate was then treated 3 times by the Sevag deproteinization method, employing chloroform and amyl alcohol. The still turbid lysate was then treated with 5 volumes of alcohol, in which a fibrous precipitate formed. The precipitate was allowed to remain overnight in the alcohol to assist the process of protein denaturation and to inactivate traces of pneumococcal desoxyribonuclease. The fibrous material was then drained of alcohol, and redissolved in saline for repeated deproteinizations. The usual preparation received a total of 9 treatments with chloroform and amyl alcohol, and 3 alcohol precipitations. The final solutions in saline were clear and viscous, their volume being adjusted from 5 to 10 cc., according to the apparent bulk of the precipitates obtained in the course of preparation. These solutions contained from 0.13 to 0.35 mg. per cc. of desoxyribonucleic acid as estimated by the diphenylamine reaction. The extracts were sterilized by alcohol precipitation and stored as sterile saline solutions. The biological activity of these solutions was tested in transformation tests in which the final concentra-

tion of desoxyribonucleic acid was in the range of 0.65 to 1.75 microgram per cc. of broth. It has been shown that as little as 0.004 microgram per cc. of Type III transforming principle induces regular transformations of R pneumococci into SIII (2). Quantitative studies were not made with the crude preparations, but titrations showed that the amounts employed for testing were well in excess of the minimum amount of extract required to obtain transformation.

The Transformation Tests

In performing transformation tests, 2 cc. of broth containing the necessary accessory serum constituents, noted above, was pipetted into small sterile tubes. The broth, described previously (2), was adsorbed with charcoal prior to its use. Transformation tests were made in quadruplicate or quintuplicate, each tube containing the same amount of transforming principle. The latter was added to the tubes by diluting in saline a small sample of the stock solution of the transforming principle to be studied, so that 0.1 cc. contained the desired amount of desoxyribonucleate. A tenfold dilution of the stock solution was usually employed. In each experiment one or more control tubes were provided, to which no transforming principle was added. All tubes were inoculated with 0.05 cc. of a 10^{-4} dilution of a 4 to 8 hour blood-broth culture of the strain being transformed, unless otherwise noted. Transformation tubes and controls were incubated from 16 to 24 hours at 37°C. At this time preliminary readings could be made in certain of the transformation systems where the transformed bacteria and their progeny grew diffusely in the supernate. This is typical of the R to S transformations. Some of the transformations to be described do not give clear readings of this kind, since in some cases the transformed cells were also agglutinated by the same agent used to agglutinate the inoculated strain. In every experiment samples of the populations in each of the tubes were withdrawn for examination by streaking a loopful of culture upon a sector of an agar plate. Upon incubation, these samples produce about 100 to 200 well isolated colonies. Conclusions concerning the composition of the populations after transformation were based upon a study of the colonies in these subcultures.

Strains of Pneumococci Studied

In the present study the classical rough races will be called "R," while the classical smooth races will be referred to as "S." Since the S races can be of various serological types, the Roman numeral corresponding to the serological classification of the race will be added to classify the strain more precisely. For example, the Type III pneumococcus used in the present study will be referred to as SIII. Implicit throughout the present report will be the assumption that the salient difference between R and S races is the fact that the individual S bacterium possesses a specific transforming principle which is lacking in R pneumococci.

The following strains of pneumococci have been used in the course of the experiments to be described:—

1. Strain A69, a Type III smooth strain, used for preparation of Type III transforming principle. (Colonies of this strain are illustrated in Fig. 5.)

2. Strain B34A, a rough strain derived originally from a Type II smooth strain, D32. This strain has two characteristics of importance in transformation experiments. Bacteria of this strain can be transformed to any of several different types of smooth strain under the action of the appropriate transforming principles (4). Furthermore, no spontaneous reversions to the rough condition have ever been observed to occur, although the strain has been repeatedly subjected to conditions favorable for the detection of such reversion. This strain has been used both as a source of transforming extracts and as a test organism for the transforming activity of various extracts. (Fig. 2.)

3. Strain B34B (variant), spontaneous mutation from strain B34A. This variant has an extremely rough colony form, and in liquid culture grows in aggregates. It has been used

both as a source of desoxyribonucleate, and as a test organism for transforming activities of various extracts. (Fig. 1.)

4. Strain SIII-1, a mutant Type III pneumococcus which appeared spontaneously in an old refrigerated culture of an SIII line. This latter line had been established from a single colony of strain R36A transformed to the SIII condition, under the action of an SIII transforming principle isolated from strain A66. (Fig. 3.)

5. Strain SIII-2, a mutant Type III pneumococcus which appeared in a transformation experiment in which strain R36A was being transformed under the action of SIII transforming principle isolated from strain A66. (Fig. 4.)

These last two strains have also been used both as organisms for testing transforming activities of various extracts, and as sources of transforming principles.

Experiments with the ER Strain

Strain ER (extreme rough) can revert to the R condition. After a few serial passages of ER in ordinary liquid culture, appreciable numbers of R pneumococci may be found. On solid media populations of ER pneumococci appear to be completely stable. Thus, the instability of strain ER in liquid media can be attributed to selective forces favoring the growth of the diffusely growing R bacteria. Strain ER can, however, be maintained in pure condition in liquid culture if it is grown in a shallow layer of broth in an Erlenmeyer flask. Highly aerobic conditions are not favorable for the growth of pneumococci, and apparently growth in shallow layers is relatively more unfavorable for the diffusely growing R bacteria than for the ER bacteria, which presumably establish localized, less aerobic, areas. After cessation of growth in these shallow layer cultures, ER pneumococci must be transferred into small sterile test tubes for preservation, for like other pneumococci, these cannot be preserved under highly aerobic conditions.

The characteristic colonies of strain ER are shown in Fig. 1.

Transformation of ER to R.—Transformations of the ER strain were undertaken in the absence of added R agglutinins, since the ER bacteria grow spontaneously in an aggregated state. Serum factor in the form of bovine serum albumin was added to nutrient broth, as well as transforming principle obtained from SIII pneumococci. Experiments were carried out in the usual manner, using a volume of 2 cc. of medium placed in small test tubes. The tubes were inoculated with 0.05 cc. of a 10^{-3} dilution of an 8 hour culture of ER, grown in a shallow layer.

In this transformation system no SIII organisms were found after the usual incubation period. However, the populations of pneumococci in all the tubes which received the transforming principle were found to be predominantly R pneumococci. In an occasional control tube containing no added transforming principle, appreciable numbers of R pneumococci were also found. Since the ER pneumococcus can give rise spontaneously to R forms, two hypotheses could be offered to explain the invariable presence of the large numbers of R pneumococci in the tubes which had received the transforming principle. Either this desoxyribonucleic acid preparation was acting as a powerful selec-

tive agent for spontaneously formed R pneumococci, or this material was acting as a specific transforming agent as it is known to do in transformations of R pneumococci into SIII.

Experiments disclosed the following facts, and clarified the mode of action of the desoxyribonucleic acid fraction of the SIII bacteria upon the ER strain.

1. The predictable appearance of large numbers of R pneumococci in cultures of the ER strain depends upon the presence in the environment of adequate concentrations of the desoxyribonucleic acid fraction from suitable pneumococci. A titration of the transforming activity of this fraction of SIII pneumo-

TABLE I

Titration of the Activity of Type III Transforming Principle in Transforming Strain ER to R

Transforming preparation 55*	Quadruplicate cultures†			
Amount added	1	2	3	4
micrograms				
6.0	R	R	R	R
1.9	R	R	R	R
0.6	R	R	R	R
0.19	ER only	R	R	R
0.06	R	Few R	Few R	Few R
None	ER only	ER only	ER only	ER only

* The stock solution of transforming agent contained 0.6 mg. per cc. of desoxyribonucleic acid, estimated by the diphenylamine reaction. 0.1 cc. of appropriate dilutions were added to tubes containing 2 cc. of broth to which albumin and pyrophosphate had been added. Inoculum was 0.05 cc. of a 10^{-8} dilution of an 8 hour aerobic culture of strain ER.

† The populations were plated after 16 hours of incubation at 37°C. The symbol R indicates the presence of R colonies in addition to ER.

cocci is shown in Table I. This same highly purified desoxyribonucleate has approximately the same degree of activity in changing ER populations into R as it has in changing R populations into SIII, provided titrations are performed in the appropriate environments, both using albumin as a source of serum factor.

2. To obtain the regular production of large numbers of R pneumococci in the growing ER populations, it is necessary to add to the medium accessory serum factor (e.g. bovine serum albumin) as well as adequate amounts of the pneumococcal desoxyribonucleate.

3. Transformation of populations of ER pneumococci into R is a special property of certain desoxyribonucleate preparations. Active desoxyribonucleates are obtained from SIII and as well from R pneumococci (R36A). Highly polymerized desoxyribonucleates obtained from ER pneumococci, two

different strains of streptococci, and from calf thymus are totally inactive in this respect.

4. Small concentrations of the enzyme desoxyribonuclease (1 microgram per cc.) inhibit the action of active desoxyribonucleates in transforming populations of ER pneumococci into R, provided the enzyme is added to the transformation cultures prior to the end of the first 5 hours' incubation.

5. ER pneumococci grown for at least 5 hours in the albumin-broth mixture are capable of rapidly interacting with the transforming substance in such a way that desoxyribonuclease can no longer block the transformation into R. Hence, the ER strain undergoes the phenomenon called sensitization, described first in the studies of transformation of R pneumococci into S (6). This sensitized state, during which rapid interaction with the transforming principle is possible, is transitory for the ER pneumococci as had been found for the R, and disappears after the first 7 to 8 hours of incubation have been completed. The technique used to study the sensitization phenomenon in the transformations of the ER strain was essentially the same as described in analysis of the R to SIII transformations, and will not be discussed here.

These facts are in agreement with the interpretation that although ER pneumococci can mutate spontaneously and unpredictably to the R condition, predictable transformations are specifically induced under the action of desoxyribonucleates of certain pneumococci.

It should be noted that the same formal proof which existed in Griffith's transformation experiments (8) is not available for the case of the ER to R transformations. The essential point of the argument which established the existence of specifically induced transformation was the fact that while some R strains can spontaneously revert to the S condition, they never revert to a serological type heterologous with respect to their origin. Thus, the fact that some R strains not only could be transformed to S of heterologous type, but also to the type corresponding to the origin of the heat-killed cells used to induce the transformation, provided definitive evidence that the transformation was specifically induced. The ER strain can spontaneously revert to R, and it is the same R, as far as is known, that is produced as a consequence of induced transformation of the ER pneumococci. Furthermore, the transformation of ER into R is induced by more than one transforming extract, the desoxyribonuclease fractions of both R and SIII pneumococci being active. Hence the exclusive proof which established that the R to S transformation was specifically induced cannot be provided in the case of the transformation of ER pneumococci into R. However, the completely analogous requirements for both transformations, and the virtually identical mechanisms which operate in both cases, make it most probable that essentially the same phenomenon is occurring in both kinds of transformations; i.e., that both changes are specifically induced by an agent which is apparently a specific desoxyribonuclease.

One difference exists between the conditions necessary for the transformation of R pneumococci into S, and ER pneumococci into R. In the latter transformation rough cell agglutinins are not only not required, but actually must be

omitted. This does not imply that the agglutinated state is not required for the ER pneumococci undergoing transformation, since, as has been mentioned above, the ER strain grows spontaneously in an aggregated condition. The addition of R cell agglutinins results in a more compact aggregation of the ER bacteria, but completely inhibits the appearance of R forms in tubes which otherwise would be expected to contain more than 50 per cent R pneumococci, produced as a result of transformation of the original ER population. No SIII pneumococci were formed directly from the ER strain treated with a desoxyribonucleate of SIII bacteria, in the presence of R agglutinins. Thus, like the other morphological mutants obtained from strain R36A (2), strain ER is "incompetent" to undergo direct transformation into the SIII condition.

Two-Step Transformation of Strain ER into R into SIII.—The R pneumococci, produced by transformation of the ER strain as described above, were competent to be transformed in their turn to SIII by means of the same desoxyribonucleate, prepared from SIII pneumococci, which had induced the transformation of ER pneumococci into R. Thus, starting with the ER strain, a two-step transformation could be performed, eventually yielding SIII pneumococci. The second step, performed in the presence of R agglutinins, could readily be accomplished using any of a number of clone lines of R forms isolated from transformation experiments with the ER strain.

A single difference exists in the external conditions required by the two steps. To transform strain ER into R, R agglutinins must be omitted, while to transform R pneumococci into S, the R agglutinins must be present. It seemed possible, therefore, that in the course of a single growth cycle of an ER culture and under the action of a single dose of SIII desoxyribonucleate the two steps might be achieved if the R agglutinins were added at the appropriate time. The transformation of strain ER into R is completed after the first 5 hours of incubation (see above). Hence, in a transformation of strain ER, R agglutinins were added to some of the tubes after a preliminary incubation of 5½ hours. At the same time 2 cc. of fresh culture medium was added to prolong somewhat the growth of the cultures in the altered environment. A single addition of transforming principle obtained from SIII pneumococci had been made to all tubes at the time of inoculation with a drop of diluted ER culture. After 24 hours of incubation, a sample of the contents of each tube in the experiment was subcultured on agar plates. In all tubes to which the R agglutinins were added at 5½ hours, ER, R, and SIII pneumococci were found in the final populations. In tubes not receiving the R agglutinins, only R and ER pneumococci were found. It was possible repeatedly to obtain thus the two transformation steps in a single culture of strain ER, and with a single portion of transforming principle, by supplying the R agglutinins at the proper time.

When strain ER was transformed under the action of a desoxyribonucleate obtained from the R strain R36A, the sole transformation products were R cells, irrespective of the addition of the R agglutinins after 5½ hours' incubation.

This is in agreement with the previously reported findings that this R strain contains no transforming principle inducing synthesis of specific polysaccharide (3). It therefore appears that the desoxyribonucleate fractions of both R and SIII pneumococci have one transforming principle in common, that which transforms strain ER into R, while this fraction of the SIII pneumococci contains a second transforming principle as well, that which transforms R pneumococci into SIII. The analogous fraction of strain ER pneumococci has neither transforming activity, and presumably neither of the two transforming principles which appear to exist in the desoxyribonucleate fraction of the SIII organisms.

There remains to be explained why the transforming principle found uniquely in the Type III pneumococcus does not appear to act directly upon ER pneumococci. There is as yet no evidence that this transforming principle can propagate in the absence of the transforming principle found common to both SIII and R pneumococci. This latter principle, which for brevity can be called the R transforming principle, can on the other hand be propagated in the absence of the SIII transforming principle, for that is its condition in the rough strain used for these transformation studies. Thus, the mutually independent existence of these two transforming principles as genetic units is not demonstrated by the reported experiments. It is possible that the action of the SIII transforming principle upon the pneumococcal cell is dependent upon the phenotypic activities of the R transforming principle. Until this is shown to be the case, or until pneumococci are found which possess the S transforming principle in the absence of the R principle, strict proof that the desoxyribonucleic acid fraction of SIII pneumococcus contains at least two distinct entities controlling hereditary traits cannot be offered.

Transforming Principles Present in the Transformed Pneumococci.—Each step of the two-step transformation just described produced pneumococci which were permanently altered with respect to the newly acquired character. From R pneumococci obtained by transformation of ER forms, and from the stepwise produced SIII pneumococci, active transforming principles were isolated. The transforming extracts of the R strain produced by transformation of the ER strain possessed only the capacity to transform ER pneumococci into R. The transforming extracts of the SIII cells produced by two-step transformation of ER possessed both the capacity to transform ER pneumococci into R, and R into SIII. Hence, the properties of the transforming extracts of the doubly transformed pneumococci reflected both steps of the two-step transformation, and the pneumococci thus produced appeared to have acquired two transforming principles in the course of the two transformation steps.

The Intermediate SIII Strains

Between the colony forms S and R there exists a spectrum of gradations. Colonies having an appearance intermediate between R and S are produced

by stable strains of pneumococci which agglutinate to varying degrees in both type-specific and group-specific antisera (9, 5). Usually S pneumococci are not agglutinated by group-specific antisera unless steps have been taken to remove the polysaccharide capsule. The intermediate forms have rarely been found to be virulent, but they have been found to be capable of reverting to the virulent S condition. Such intermediate forms have been obtained by growing smooth pneumococci in homologous specific antiserum. Very commonly, however, intermediate forms are encountered in old, refrigerated S cultures, indicating that the antiserum is not essential for their production.

The mutant SIII strains to be discussed are two of a number of spontaneously appearing variant SIII pneumococci which have been encountered over a period of 2 years' work. Their origin has been noted above.

Strain SIII-2 produces a mucoid colony on solid medium, but the colony is distinct from the mucoid colonies of the SIII strain, A66, and of R pneumococci transformed by the SIII transforming principle isolated from strain A66. The colony of SIII-2 is smaller, dries out more rapidly, and autolyzes sooner than do the colonies of the latter two strains. In general the colonies of the SIII strains isolated from nature are more mucoid than those of other smooth types. The strain SIII-2 thus resembles superficially other smooth races of pneumococci more than it does the parent SIII line from which it was indirectly derived. The variant is, however, serologically a Type III organism, and gives a quellung reaction (swelling of the capsule) in Type III antiserum. SIII-2 is capable of establishing infection when relatively small numbers of organisms are injected intraperitoneally into mice. See Table II. The strain is stable in liquid culture, but is capable of giving rise to rare mutants which produce colonies like those of the normally mucoid SIII strains.

Strain SIII-1 is not mucoid, and its colony can be distinguished, with difficulty, from an R colony. Although by agglutination tests SIII-1 must be classified as a Type III organism, it does not give a quellung reaction in Type III antiserum. Nor is strain SIII-1 virulent, although it is capable of reverting to the normally mucoid, virulent condition. See Table II. The SIII-1 variant is analogous to the variant of an SII strain described by MacLeod and Krauss (5).

Colonies of these strains are shown in Figs. 3 and 4.

Serological Characterization of the Variants.—The various races of S pneumococci are differentiated by their capsular antigens, the specificity of which depends upon the chemical composition of the capsular polysaccharides. However, S pneumococci as well as R pneumococci possess a large number of somatic antigens which appear to be common to the group *Pneumococcus*. Thus, a distinction is made between the group-specific antigens common to encapsulated and non-encapsulated forms alike, and the type-specific antigens, found only in the encapsulated races. As far as is known, each S race forms only one kind of capsular polysaccharide, or type-specific antigen.

Antisera resulting from the injection of R pneumococci into animals possess antibodies directed against the group-specific antigens, while antisera against S forms usually contain antibodies which react with both group-specific and type-specific antigens. Thus, an antiserum prepared with an SIII strain is usually capable of agglutinating both R and SIII strains, but an antiserum prepared with an R race is incapable of agglutinating the usual S races. An antiserum containing type-specific antibody only, which reacts only with homologous polysaccharide or organisms, can be made by adsorption of a given antiserum with heat-killed R pneumococci.

Using antisera made with normal Type III pneumococci, and antisera against strain R36A, an analysis was made of the antigenic structure of the SIII races

TABLE II

Results of Intraperitoneal Injection of Mice with 8 Hour Blood-Broth Cultures of the Intermediate Smooth Strains

Infective dose	Strain SIII-2		Infective dose	Strain SIII-1	
	Result	Organism recovered		Result	Organism recovered
cc.			cc.		
10^{-1}	D 30 hrs.	SIII-2	0.5	D 24 hrs.	SIII-1
10^{-2}	D 24 hrs.	SIII-2	0.5	D 24 hrs.	SIII-1
10^{-3}	S		0.5	D 48 hrs.	SIII-N
10^{-4}	D 36 hrs.	SIII-2	0.5	S	
10^{-5}	S		0.5	S	
10^{-6}	D 36 hrs.	SIII-2	0.5	S	

D = dead.

S = survived.

used in the present study. The essential points shown by these studies are the following:—

1. The normal SIII strain, to be referred to henceforth as SIII-N, and mutant strain SIII-2 are agglutinated only by antiserum containing antibody reacting with the Type III polysaccharide (Tables III and V).

2. Strain SIII-1, the non-mucoid variant, and also strain R36A, an R strain, agglutinate in antisera containing group-specific antibody (Table V). Larger quantities of antibody are required to agglutinate SIII-1, than are required to agglutinate the R strain.

3. Strain SIII-1 is also agglutinated by Type III type-specific antibody. This reaction is revealed in Type III antipneumococcal serum adsorbed with R bacteria to remove the group-specific antibodies normally present in such sera (Table IV).

4. Less Type III antibody is required to agglutinate intermediate strains SIII-1 and SIII-2, than is required to agglutinate the fully mucoid SIII-N strain (Tables III and IV).

5. When strains SIII-1, SIII-2, and SIII-N are grown in the presence of group-specific antibody and the enzyme which hydrolyzes the Type III capsular polysaccharide, it is found that the enzyme uncovers the group-specific antigens of all three strains. Strains-SIII-N and SIII-2, which normally agglutinate only in the presence of adequate amounts of Type III antibody, are so altered by the enzyme that they can be agglutinated by R antibody, or group-specific antibody. Similarly, through the action of the enzyme, SIII-1 pneumococci are rendered sensitive to much smaller amounts of R antibody than are otherwise required to agglutinate this strain. Thus, the enzyme which splits

TABLE III

Thread Test Reactions of SIII-N Cells and SIII-2 Cells in Gamma Globulin Fraction of Type III Immune Horse Serum

Antigen	Dilution of antibody solution in broth									
	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120
SIII-N	++++	++++	++	++	+	-	-	-	-	-
SIII-2	++++	++++	++++	++++	++++	++++	++	+	+	-

Serum fraction contained rough agglutinins but neither strain reacted with rabbit R agglutinins (*cf.* Table V). +++++, complete sedimentation of growth. ++++, slightly granular supernate. ++, bulk of growth in granular supernate. +, finely granular supernate, no sedimented growth.

TABLE IV

Agglutination Reactions of SIII-N and SIII-1 Cells in Type III Immune Rabbit Serum

Antigen	Dilution of antibody solution in 0.85% sodium chloride										
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048
SIII-N	++++	++++	++++	++++	+++	+++	++	+	-	-	-
SIII-1	++++	++++	++++	++++	+++	+++	+++	+++	+++	++	-
RVA	+	+	-	-	-	-	-	-	-	-	-

Serum agglutinated with RVA to remove rough cell agglutinins. Read as in Table III.

the Type III polysaccharide attacks also the type-specific antigens of the mutant SIII strains (Table V).

These experiments indicate that the mutant strains each form Type III polysaccharide. It should be emphasized that while serological cross-reactions are found between a number of polysaccharides of pneumococcus, none of the polysaccharides which show serological cross-reactions with the Type III polysaccharide can be split by the enzyme used to hydrolyze this latter polysaccharide in the present experiments. Thus, the data just summarized support the view that the polysaccharide formed by the SIII mutants is unaltered in chemical composition from that formed by the normal Type III strain.

Eight mice were injected with living SIII-1 cells from 1 cc. of an 8 h. ur

blood-broth culture, on 4 successive days during each of 2 weeks. 7 days following the last injections two sera were obtained which reacted down to concentrations of 1:64,000 and 1:16,000 of purified Type III polysaccharide respectively.

Attempts were made to adsorb the type-specific antibody from a Type III immune serum by means of cells of the non-mucoid variant. 4 cc. of a diluted serum was adsorbed with the variant cells obtained from 1500 cc. of culture.

TABLE V

Thread Tests with the Three SIII Strains, in Serial Dilutions of a Concentrated Gamma Globulin Fraction of Serum from Rabbits Immunized with R Strain R36A

Series A								
Antigen	Dilution of antibody solution in broth							
	1/40	1/80	1/160	1/320	1/640	1/1280	1/2660	1/5120
SIII-N	—	—	—	—	—	—	—	—
SIII-2	+	—	—	—	—	—	—	—
SIII-1	+++	+++	+++	+++	++	++	+	—
R36A	++++	++++	++++	++++	++++	++++	++++	++++

Series B							
Antigen		Dilution of antibody solution in broth					
		1/250	1/500	1/1000	1/2000	1/4000	1/8000
SIII-N	Enzyme-hydrolyzing SIII polysaccharide present in constant concentration	+++	+++	++	++	++	
SIII-2			++++	++++	++++	++	
SIII-1		++++	++++	++++	++++	++++	
R36A		++++	++++	++++	++++	++++	++++

In series A, antibody solution was diluted with broth. In series B, antibody solution was diluted with broth containing the enzyme which hydrolyzes Type III polysaccharide. Read as in Table III.

As a result of this adsorption a fourfold decrease was observed in the agglutination titer of this serum with normal SIII cells. The SIII-1 cells had removed some of the Type III agglutinins from the serum, but left behind considerable activity. The residual specific antibody, like the unadsorbed antibody, agglutinated SIII-1 bacteria at higher dilutions of antiserum than it did the normal SIII cells, although the antibody removed had been adsorbed by the antigens of SIII-1. Repeated adsorptions led to the same result. The R antibody was completely removed, showing both that the non-mucoid variant SIII could adsorb R antibody, and that the antibody left behind was solely type-specific antibody. It can thus be concluded that the strain SIII-1 adsorbs very little of the type-specific antibody, and that repeated adsorption does not

result in a qualitative change in the type-specific agglutinins remaining in the antiserum. There is no indication from these experiments that the antigen of the SIII-1 pneumococcus differs qualitatively from the antigen of the SIII-N strains.

The purification and study of the polysaccharides of the mutant SIII strains will be necessary before it can be definitely stated that normal and mutant strains are producing exactly the same polysaccharide. The data thus far obtained are in harmony with the view that each of the organisms studied is capable of synthesizing the Type III polysaccharide, and that the differences between the strains lie in the rates at which the polysaccharide is formed or released at the surfaces of the bacteria. The obvious morphological differences between the colonies of the SIII strains suggest that the quantity of polysaccharide is less in strains SIII-2 and SIII-1 than in the normal SIII strains. There are two other reasons for believing that the differences between the SIII strains are quantitative.

1. It is clear that SIII-2, SIII-1, and SIII-N strains are agglutinated by different quantities of Type III antibody. The non-mucoid SIII-1 strain requires the smallest amount, SIII-2 requires more, while SIII-N requires the most. Since the Type III polysaccharide is water-soluble, antibody is neutralized by polysaccharide in solution around the bacteria, and hence is neutralized without contributing to the agglutination of the bacteria. Thus, the strain releasing the smallest amount of polysaccharide in solution would be expected to be agglutinated by the smallest quantity of specific antibody.

2. The three strains differ with respect to the ease with which the same concentration of polysaccharide-splitting enzyme can free the bacterial surfaces of specific polysaccharide (Table V). Although the enzyme was prepared by adapting *B. palustris* so that it could utilize as a carbon source the capsular polysaccharide of the normal Type III pneumococcus, the enzyme is more effective in uncovering the non-specific antigens during growth of strains SIII-1 and SIII-2 than in uncovering these antigens in cultures of the normal SIII strain. This hydrolytic enzyme does not destroy the capacity of the pneumococcal cell to form Type III polysaccharide, and whether or not the non-specific surface antigens are uncovered during growth in the presence of the enzyme depends very likely upon the balance existing between the rate of destruction of the polysaccharide and the rate of its synthesis or release at the bacterial surface.

The Nature of the Altered Heredity of SIII-2 and SIII-1.—The SIII-N bacterium differs from the R in possessing the Type III transforming principle, which will be referred to as the SIII-N transforming principle. The question was therefore raised whether the mutant SIII strains possessed the same transforming principle or some analogous transforming principle.

Extracts possessing transforming activity were obtained from both of the intermediate smooth strains. With these extracts the susceptible R strain

R36A could be transformed to the SIII-2 or the SIII-1 condition according to the origin of the extract used. Each extract conferred upon the R pneumococcus the ability to synthesize the type-specific antigen, and as well, determined that the transformed cell formed the antigen in a particular altered condition. In no case did either extract transform R bacteria to the fully mucoid condition, nor was it ever observed that an extract from SIII-2 transformed R36A to SIII-1, or *vice versa*. Each extract possessed a strictly specific transforming action. The intermediate Type II strain studied by MacLeod and Krauss (5) likewise appears to contain a transforming agent which transforms R pneumococci into the intermediate Type II state. The results of these authors are analogous with the results just reported here.

A highly purified desoxyribonucleate was prepared from SIII-1. The extensive purification did not alter qualitatively its transforming activity. This desoxyribonucleate fraction transformed R36A to SIII-1 and also transformed strain ER into R. Hence, like the SIII-N nucleic acid it appeared to possess two transforming principles, each having a specific activity.

Rough strains were derived from the mutant strains SIII-1 and SIII-2 by serial passage of these strains in 10 per cent Type III immune serum. Both of these rough strains proved capable of being transformed to SIII-N under the action of the SIII-N transforming principle. This indicated that the hereditary properties of the mutant SIII strains permitted normal antigen synthesis provided the normal SIII transforming principle was possessed by the bacteria. This fact taken in conjunction with the isolation of transforming agents having altered activity indicates that it is the SIII-N transforming principle itself which has undergone some alteration in the mutant S strains.

Although phenotypically the SIII strains appear to differ from each other in a quantitative way, no evidence was found to indicate that the apparently quantitative variation of capsular polysaccharide formation was due to a variation of the quantity of SIII-N transforming principle, common to all of the SIII strains but present in different amounts. This may be seen from the following considerations.

If the variations in polysaccharide synthesis were due to the presence of different numbers of units of the Type III transforming principle in the various races, one would expect transformations of the R strain by means of the SIII-N transforming principle to yield a spectrum of Type III forms. Detailed examination of the kinds of smooth bacteria produced by such transformations showed that almost invariably normal Type III bacteria were produced. As a very rare exception this transforming principle will induce the production of variant smooth pneumococci. These anomalies have been of two sorts. Several lines of SIII pneumococci have been recovered, each characterized by apparent quantitative modification of polysaccharide synthesis. Strain SIII-2 is one such line. In addition, two different lines giving smooth colonies have been found which could not be typed by means of antisera against the first 33 types of smooth pneumococci. These deviations in the results of transformations are extremely rare, however, and have the same degree of unpredictability as has the phenomenon of mutation. Thus, it is concluded that just as no unit comparable to the normal Type III transforming principle exists in the desoxyribonucleic acid fractions

of the intermediate SIII strains, likewise no units comparable to the mutated Type III transforming principles seem to exist in the desoxyribonucleic acid fraction of the normal SIII pneumococci.

This conclusion is strengthened by the results of the following experiments. R pneumococci were transformed under the action of mixtures of equal amounts of the SIII-N transforming principle and either one of the mutant SIII transforming principles. Under these conditions, smooth strains corresponding to each of the transforming principles were recovered simultaneously in the transformed populations. Quantitative studies of the proportions of each kind of smooth pneumococcus strain were not made, because of the difficulties in assessing the rôle of selection in determining the final composition of the populations. However, the proportions recovered were approximately equal, when approximately equal amounts of each transforming agent were introduced. The results of these experiments can most simply be interpreted by supposing that each of the SIII transforming principles is a distinctive entity equally effective in competing in reactions with the sensitized R pneumococci.

Additive Effect of the Mutant SIII Transforming Principles

Each SIII transforming principle proved to be strictly specific in its action upon the R pneumococcus when it was introduced singly into the transforming system, or in experiments where the SIII-N principle was introduced simultaneously into the transforming system with either one of the mutant principles. However, when mixtures of the two mutant principles, SIII-1 and SIII-2, each in excess of the amount necessary to produce transformations, were introduced into the system for transforming R pneumococci, not two but three transformation products were recovered. The final population was found to contain both kinds of mutant SIII bacteria, and also normal SIII-N bacteria. Few of these SIII-N pneumococci were found, but their appearance is significant in view of the failure to obtain any such forms when the mutant transforming principles were added singly to this transforming system. The appearance of the SIII-N bacteria indicates that some form of summation can take place between the two mutant principles in their action upon a sensitized R pneumococcus, resulting in normal polysaccharide synthesis.

Nonetheless, it is clear that a simple quantitative difference between the SIII transforming principles does not exist. The SIII-2 agent used in excess of the amount necessary for transformation never yielded this summation; nor did the SIII-1 transforming principle. The existence of the interaction just described is, therefore, evidence that each of the mutated principles differs from the SIII-N principle in some unique respect.

Two-Step Transformations of R into SIII-1, into SIII-N

A turbid pneumococcus which has been transformed to the lowest degree of opalescence by means of the principle obtained from SIII-1 bacteria can again be transformed, under the action of the SIII-N principle in an appropriate concentration. SIII-1 pneumococci are poorly agglutinated by R agglutinating solution; therefore, it was necessary to provide a better means of agglutina-

tion. The techniques used have been described in detail above. Briefly, agglutination was achieved either by adding to the usual transforming system containing R antibody the enzyme which hydrolyzes the Type III polysaccharide, or by replacing the R agglutinins with SIII agglutinins. Although these techniques yielded completely agglutinated growth of the SIII-1 inoculum, the SIII-N cells produced by transformation had a tendency to escape the agglutinating mechanism. Thus, in tubes in which transformation occurred, the supernatant medium often contained granular or even diffuse growth.

In these systems, massive and regular transformations of SIII-1 populations to SIII-N were obtained. However, since the SIII-1 cells possess the capacity to mutate spontaneously both to the SIII-N condition and the R condition, three mechanisms may be conceived by which the SIII-N pneumococci could be formed. First, they could be formed by directly induced change of SIII-1 bacteria into SIII-N through the action of the SIII-N transforming principle. Second, they could appear through the selection of spontaneously occurring SIII-N mutants. Third, they could be produced by a transformation of the rare R mutants of the SIII-1 strain, through the action of the SIII-N transforming principle. Experiments were therefore done to determine how the SIII-N cells were produced in this transforming system. The following facts were ascertained:—

1. The regular appearance of SIII-N pneumococci is dependent upon the presence of adequate quantities of the SIII-N transforming principle. Titrations of the action of this purified desoxyribonucleate fraction of SIII-N pneumococci upon the SIII-1 population indicated that its activity was of the same general magnitude in this transformation and in transformations of R into SIII-N. In general, transformations of SIII-1 pneumococci into SIII-N occurred at slightly lower concentrations of a given preparation of SIII-N transforming principle than was needed for transformations of R into SIII-N. The significance of this greater sensitivity of the SIII-1 bacteria in the transforming system is not understood at present. Table VI shows a titration of an SIII-N transforming principle in both transformation systems. Both titrations were performed the same day, and using the same reagents. This pair of titrations shows an extreme manifestation of the differences in activity of the same desoxyribonucleate fraction in the two transformation systems.

2. Spontaneous mutation of SIII-1 to SIII-N has a negligible rôle in these experiments. Of 66 control tubes which did not receive transforming principle, only 1 was found to contain enough SIII-N pneumococci to be manifest upon plating.

3. The accessory serum factor is essential to obtain the regular and massive changes characteristic of the transformations of SIII-1 populations into SIII-N.

4. By use of the enzyme desoxyribonuclease it was found that in the transformation of SIII-1 into SIII-N the sensitization phenomenon occurred in a fashion exactly parallel to that already observed for strains R36A and ER.

The sensitization of SIII-1 pneumococci is achieved after a period of $4\frac{1}{2}$ to 5 hours' incubation. After this time an exposure of as little as 5 minutes to the transforming principle is adequate to induce massive changes of the SIII-1 population into SIII-N.

The high degree of control over the outcome of the transformations of SIII-1 pneumococci into SIII-N precluded the possibility that rare spontaneous mu-

TABLE VI

Comparative Titrations of the Activity of Desoxyribonucleic Acid Fraction of Type III Pneumococcus in Transforming R Cells into SIII-N and SIII-1 Cells into SIII-N

Transforming Preparation 55	Quadruplicate tests*			
Micrograms added	Inoculum—R36A		Agglutinins—R antibodies	
	1	2	3	4
6.0	SIII-N	SIII-N	SIII-N	SIII-N
1.9	SIII-N	SIII-N	SIII-N	SIII-N
0.6	R	R	R	SIII-N
None	R	R	R	R
Transforming Preparation 55	Inoculum—SIII-1		Agglutinins—SIII antibodies	
	1	2	3	4
0.6	SIII-N	SIII-N	SIII-N	SIII-N
0.06	SIII-N	SIII-N	SIII-N	SIII-N
0.006	Few SIII-N	SIII-N	Few SIII-N	SIII-N
0.002	Few SIII-N	Few SIII-N	Few SIII-N	No SIII-N
None	SIII-1	SIII-1	SIII-1	SIII-1

Dilutions of transforming principle were added in 0.1 cc. amounts to tubes containing 2 cc. of broth to which albumin and pyrophosphate and agglutinins had been added. Inoculum was 0.05 cc. of a 10^{-4} dilution of a 4 hour blood-broth culture of the strain designated.

* The populations were plated after 23 hours of incubation at 37°C .

tation followed by selection played a critical rôle in changing the SIII-1 population into SIII-N. However, it could be excluded on experimental grounds that the appearance of the SIII-N bacteria in the treated populations of SIII-1 bacteria was due to the transformation of rare R variants in the SIII-1 strain. It was observed many years ago that type-specific antisera inhibit the transformation of R36A into SIII-N (McCarty and Avery¹). This inhibitory action is not confined to Type III antisera. In the present experiments, complete transformation of R strain R36A into SIII-N was obtained with

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the same solution of Type III antibody which provided essential environmental conditions for the transformation of SIII-1 into SIII-N.

These experiments have led to the conclusion that SIII-1 bacteria can be directly transformed into the SIII-N condition through the action of the SIII-N transforming principle, under the appropriate environmental conditions. While the *in vivo* experiments of MacLeod and Krauss (5) have suggested that a Type II intermediate form could be transformed into a normal Type II organism under the action of a normal Type II transforming principle, the evidence offered by these workers does not appear adequate to prove that the intermediate form itself was the organism undergoing transformation. A possible rôle of R pneumococci, formed by spontaneous mutation of the intermediate strain, might be adduced to explain the results of the cited authors. The *in vitro* techniques used in the present study upon the SIII races provide evidence which rather clearly indicates that a pneumococcal cell, already endowed with the capacity to synthesize a specific polysaccharide, can be transformed under the action of a transforming principle inducing the formation of a different (or more of the same) specific polysaccharide.

The SIII-1 pneumococcus could also be transformed to a Type II pneumococcus by means of a desoxyribonucleate fraction of Type II pneumococci. Studies upon these transformations will be reported in detail at some future date.

When the R pneumococcus was transformed first to the SIII-2 condition, further transformation to the SIII-N condition under the action of the SIII-N transforming principle was not found possible. Likewise, when the R pneumococcus was transformed first to the SIII-N condition, the SIII-N forms produced could be transformed neither to the SIII-2 nor to the SIII-1 condition. The SIII-2 and SIII-N principles induce the formation of large amounts of capsular polysaccharide, which may interfere with the sensitization process. This possibility should be kept in mind, as well as the possibility that the failure to obtain these transformations is due to some properties of the transformation principles themselves.

Mechanism of the Two-Step Transformation, R into SIII-1, into SIII-N.—The SIII-N bacterium produced by this two-step transformation has reacted with two distinct transforming agents. An investigation was therefore made to see whether the desoxyribonucleate fraction of this doubly transformed bacterium contained both the SIII-1 and SIII-N transforming principles.

Special techniques were employed to detect SIII-1 pneumococci in the final populations of these transformation experiments, since the SIII-1 colony is difficult to distinguish from an R colony. When cultures are made by mixing 100 to 200 pneumococci in liquid nutrient agar, just before pouring the plate, plates are obtained in which colonies are well separated and submerged. If blood is omitted from the agar, and a potent homologous type-specific antiserum added before plating, around each smooth colony a halo will appear after 48 hours of incubation. This halo results from the reaction between the diffusing soluble capsular polysaccharide and the type-specific antibodies in the agar. R pneumococci produce no halo. SIII-N

pneumococci produce halos several millimeters in diameter, which stand well out from the colonies. Between the ring of precipitate and the colony is a zone of clear agar, since the large amount of polysaccharide produced by this strain redissolves the polysaccharide-protein precipitate (prozone phenomenon). This halo travels centrifugally as incubation is prolonged. SIII-1 pneumococci, however, form small halos which show no discontinuity between the precipitate and the colony. By plating suitable dilutions of the populations produced by transformation of strain R36A with the transforming extracts described in the following paragraph, the population could readily be examined for the presence of SIII-1 pneumococci.

Nucleic acid extracts were made from the progeny of a single colony isolate of an SIII-N pneumococcus produced by two-step transformation of strain R36A. Also, an extract was made from the progeny of a mixed culture established from a large number of SIII-N colonies obtained directly from the plated population of one of these two-step transformations. With both preparations results were identical, and when their action on strain R36A was studied, only SIII-N and R colonies were found on the plates made from the final population. There was, thus, no indication that these extracts could transform strain R36A into SIII-1.

The SIII-N strains produced by two-step transformation of R show no signs therefore of having once been in contact with and transformed by the SIII-1 transforming principle. It is as though in the two-step transformation of R into SIII-1 into SIII-N, the SIII-1 transforming principle has been, in effect, destroyed by the second transformation step. Hence the second step of this two-step transformation may consist of an exchange of the SIII-1 transforming principle in the bacterium with the SIII-N principle in the environment; or the second step may consist of some kind of interaction between the two principles, in which the SIII-1 principle loses its independent existence.

Analysis of the Specificity of the SIII-2 Transforming Principle in the Two-Step Transformation of R into SIII-1 into SIII-2.—Two-step transformations could be done by inducing the transformation of R pneumococci into SIII-1, by means of the appropriate principle, and following this, transforming the SIII-1 pneumococci under the action of the SIII-2 transforming principle. As has been indicated, two distinct transformation systems are necessary for the two steps, since SIII-1 pneumococci are poorly agglutinated by R antibody.

At the end of the second step, the final population of pneumococci found in the tubes which had received the SIII-2 transforming principle was composed of three kinds of pneumococcal cells. First, there was the residue of untransformed SIII-1 bacteria; second, there were large numbers of SIII-2 bacteria; third, there were small numbers of SIII-N forms. In the control tubes, receiving no transforming principle, only SIII-1 forms were found. Thus, under the action of the SIII-2 transforming principle, two transformation products were produced from the SIII-1 bacteria inoculated.

It should be emphasized that the SIII-2 transforming principle, acting upon R pneumococci, induces the formation of SIII-2 pneumococci only. Thus, here, as in the experiments cited above in which the SIII-1 and SIII-2 trans-

forming agents were introduced simultaneously into the transformation system of strain R36A, it appears that some form of summation between the two mutant principles can occur if the SIII-2 principle is interacting with a pneumococcus already possessing the SIII-1 principle. Summation does not always occur between these two principles. This is clear from the fact that the majority of new kinds of pneumococci produced in the transformation of the SIII-1 forms by the SIII-2 principle are SIII-2 forms.

This effect of the SIII-2 principle, when acting upon the SIII-1 pneumococcus, suggests again that transformation can be either an exchange reaction (producing SIII-2 forms), or some form of interaction (producing SIII-N forms) with the SIII-1 principle already possessed by the bacterium undergoing the transformation.

DISCUSSION

Transforming activity may be regarded simply as a new kind of character and it is possible that the transforming agents themselves are better suited to elucidate bacterial heredity than the more immediately accessible characters of bacterial cells, such as enzymatic constitution or antigenic structure. Transforming agents appear to be concerned with the heredity of pneumococcus in much the same fashion that genes are concerned with the heredity of higher organisms. Thus, they appear to have autocatalytic and heterocatalytic functions, and play a decisive rôle in determining the properties of a given pneumococcus and its progeny. It is, of course, not clear whether a transforming principle is a complete genetic determinant, since the transformed cell contributes the necessary environment for the expression of its activity. However, it is clear in the case of the R into SIII transformations that the transforming extracts include the constituent of the genetic system which is essential to the synthesis of a capsular polysaccharide, and which determines the chemical nature of this polysaccharide. Strictly speaking, very little more can be said even of genes with reference to their relationship to the characters they determine.

Certain aspects of the present experiments suggest further that the transformation technique may, in fact, be generally applicable to the study of the mechanism of heredity in the pneumococcus. The following points may be cited:—

1. The discovery of a new transforming principle, active in transforming ER cells into R, provides evidence that more than one character of the bacterium is under the control of a transforming principle having the properties of a desoxyribonucleate.

2. Evidence presented indicates that the desoxyribonucleate fraction of the SIII bacterium contains a minimum of two transforming principles. Even though complete evidence has not yet been obtained to the effect that the two transforming activities possessed by this fraction can be ascribed to two com-

pletely independent genetic units, such an assumption remains the most plausible.

3. Application of transformation techniques in a study of two mutated races of SIII pneumococcus showed that the SIII transforming principle itself had mutated in these races, giving rise to the mutant properties of these strains. Mutation of other hereditary factors in the altered strains which might result in modified polysaccharide synthesis, could be ruled out. Thus, by the transformation study it has been possible for the first time to determine that a mutated bacterium differs from its normal progenitor because of a spontaneous alteration which has occurred in a given entity possessing genetic activity. Further, it could be seen that it was the same entity which had undergone mutation in each of the mutated races.

The present study differs from preceding ones because the mutation of the SIII-N strain produced forms in which a mutated SIII transforming principle could be demonstrated. R forms also may contain a mutated S transforming principle, the heterocatalytic activity of which is not obvious.

4. The R transforming principle which induces the ER to R transformation is found in the desoxyribonucleate fractions of both the R and S bacteria thus far studied. Hence, although the R-ness of the S pneumococcus is not visible in its colony morphology, it can be demonstrated that the R principle is nonetheless present in the S pneumococcus. This kind of information about the genetic constitution of the S bacterium is analogous to that obtained in classical genetics when the experimenter demonstrates the presence of genes whose phenotypic manifestations are in some way masked.

This last consideration leads to a problem of great importance in any study of hereditary systems. It is known that a gene may be masked by a dominant allele or by non-allelic genes. If transforming principles are genetic determinants, how can their relations to each other be assessed? For example, are the R and S transforming principles allelic or not?

Clearly, if allelism were to be defined strictly in terms of the techniques usually employed to discern it, allelism cannot be demonstrated in asexual organisms. If, however, it be granted that the absence of crossing-over between two genes is a secondary aspect of allelism, and that the primary property of allelism consists of the alleles having been derived from common gene ancestors, it is justifiable to consider as alleles all genetic determinants fulfilling the primary condition, even in asexual forms. It is perhaps simpler to refer to such genetic units as homologous. The problem remains how to assess the relationship between genetic units, in the absence of sexual fusion.

In the body of the present paper, observations were set forth which led to the conclusion that the SIII-N, SIII-2, and SIII-1 transforming principles are distinct, qualitatively different entities, the latter two of which have arisen by spontaneous mutation of the former. Granting that this evidence is sufficient, it is possible to look upon these transforming principles as homologous genetic

elements—or alleles, if one is content to define alleles in terms of their lineage.

It appears thus far that a given race of pneumococcus can possess at a given time only one (or none) of the SIII principles. This is particularly in evidence in the two-step transformation of R into SIII-1, SIII-1 into SIII-N. Here it is found that in the second transformation step the SIII-N principle replaces the SIII-1 principle which was readily demonstrated in the desoxyribonucleate fractions of the SIII-1 pneumococci produced by the first transformation step. This is in contrast with the simultaneous occurrence of both R and S principles in the SIII forms thus far studied. Likewise, in the two-step transformation of ER into R, R into SIII-N, the doubly transformed cell can be shown to have acquired both the R and SIII principles. This R principle has been found in the SIII races and as well in the R strain R36A, derived originally from a Type II culture of S pneumococci. Thus, there is no indication that the R principle and the SIII principles have been derived from a common ancestor-transforming principle.

From these facts it is possible at present to visualize how a distinction can be made between genetically related (homologous, allelic) transforming principles and genetically unrelated ones by means of the transformation technique. It may very well be that in successive transformations with genetically related principles, transformation consists of a replacement or interaction of the first introduced principle with the second. On the other hand, after successive transformations with two genetically unrelated principles both principles are demonstrable in the desoxyribonucleate fraction of the doubly transformed organism.

It is implicit in this hypothesis that a condition analogous to heterozygosity does not exist with respect to the transforming principles of a pneumococcus. This appears, in fact, to be the case with the SIII principles.

The SIII principles and the R principle have been referred to here as entities. This is justified by the fact that there does not seem to be any quantitative or qualitative variation in the action of any one of these transforming principles upon the appropriate pneumococcal race, except as a rare deviation. This indicates that the induction of polysaccharide synthesis, for example, results from an interaction between the susceptible R form and a qualitatively distinct unit in the desoxyribonucleate fraction of the S organisms. There is no way of knowing at present how many particles in the desoxyribonucleate solution compose each biologically complete entity. With respect to the SIII transforming principles, the experiments reported above make it highly unlikely that they differ from each other by being composed of different numbers of particles, *n* of which must be possessed in order to produce the SIII-N phenotype. However, the existence of an interaction between the SIII-2 and the SIII-1 transforming principles reacting with a given pneumococcus to produce the SIII-N form, remains to be explained. This phenomenon could most

simply be understood if the SIII-N transforming principle could be subdivided into two biologically active agents, having respectively SIII-2 and SIII-1 transforming activity. No evidence could be found that the SIII-N transforming principle is such a biparticulate entity. It is likely that to understand these experiments we will have to understand at what level of molecular organization genetic activity makes its appearance, and to what extent the very large molecules present in the transforming extracts behave as indivisible units in cell metabolism.

I am grateful to Dr. Maclyn McCarty for preparations of the enzyme desoxyribonuclease, the two streptococcal desoxyribonucleates, and the thymus desoxyribonucleate. It is a pleasure also to acknowledge the many helpful discussions of this work with Dr. O. T. Avery, Dr. McCarty, and Dr. R. D. Hotchkiss; and, in preparation of the manuscript, the extensive discussions of the genetic aspects of the transformation phenomenon, with Professor Boris Ephrussi.

SUMMARY

It has proved possible to transform an extreme rough variant of pneumococcus back to the rough state by the action of the desoxyribonucleate fractions of either rough or Type III smooth pneumococci. In a second step, the rough pneumococci produced by this transformation were further changed to the Type III smooth state by means of the desoxyribonucleate fraction of Type III smooth organisms. With use of the Type III desoxyribonucleate, the two steps could be accomplished successively, but not simultaneously, during the growth of a single inoculum of the extreme rough form.

These findings have been interpreted as indicating that the desoxyribonucleic acid fraction of Type III smooth pneumococci contains two transforming principles of differing specificity, while the same fraction of rough pneumococci contains but one of these principles.

Two distinct spontaneous variants of Type III smooth pneumococci have been isolated which seem to differ from normal Type III smooth pneumococci in synthesizing smaller amounts of the specific polysaccharide. Tests have indicated that these variant Type III races differ from the normal in possessing altered Type III transforming principles. Each of the new transforming agents when influencing rough bacteria, is strictly specific in its action, inducing as it does the formation of the corresponding variant Type III pneumococci.

Interaction between the two new transforming principles and rough pneumococci can lead to the production of normal Type III organisms, although neither principle alone can do it. This is interpreted as indicating that the two mutated Type III transforming principles are qualitatively different from each other.

Another kind of two step transformation was accomplished by converting rough pneumococci first into the variant Type III pneumococci which produced small amounts of polysaccharide, and then by transforming these latter into normal Type III organisms.

After the two-step transformation of the extreme rough pneumococcus, both transforming principles used to effect this can be recognized in the Type III smooth pneumococci finally recovered. By contrast, in the two-step transformation of the rough pneumococcus by way of an intermediate smooth form, only the second transforming principle can be obtained from the resulting fully smooth organisms.

The meaning of these facts is discussed.

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EXPLANATION OF PLATE 15

Photographed from sectors of the same blood-agar plate. $\times 40$.

FIG. 1. Colonies of the extreme rough strain, ER.

FIG. 2. Colonies of the rough strain R36A.

FIG. 3. Colonies of the intermediate smooth strain, SIII-1.

FIG. 4. Colonies of the intermediate smooth strain, SIII-2.

FIG. 5. Colonies of the SIII-N strain, A66.



Fig. 1. Blood corpuscles in the blood stream.

ELECTRON MICROSCOPE STUDIES OF CELLS BY THE METHOD OF REPLICAS*

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PLATES 16 AND 17

(Received for publication, December 15, 1948)

The technique of electron microscopy has failed thus far to find general application in the study of cells and tissues, chiefly because of the difficulties involved in the preparation of specimens of required thinness. Formed elements can be isolated from the cells and examined separately (2, 3) but in this case the connections with the other cell structures are lost and a study of the general cell organization is not possible. The use of tissue culture has offered an opportunity to examine certain portions of the cells, especially thinly extended cells margins (4), but in this case also a number of limitations must be recognized: the center of the cell, as a rule, remains too thick for study; cells in tissue culture, especially actively growing ones, have a tendency to dedifferentiate; and finally, the cellular arrangement existing in the whole organ is not retained by the outgrowing cells. It would seem, therefore, that the method of sectioning, so widely successful as an adjunct to light microscopy, would be the technique of choice because of its applicability to all cells and tissues, irrespective of their individual constitution or origin. The early attempts to adapt the method of sectioning to electron microscopy have not been completely satisfactory (5-7), and experience of recent years indicates that the problem does not reside solely in the difficulties in producing sections of sufficient thinness, but also in the fact that the current methods of fixation and embedding fail to preserve the fine structure of the cells (8-10).

As is known, the absorption and scattering of electrons are not affected by molecular differences, as is the case with light of various wave lengths, but are determined by variations in atomic densities. In biological material, therefore, where the major elements, i.e. carbon, nitrogen, and oxygen have nearly the same atomic weights, the absorption and scattering observed in the electron microscope is a measure, but in inverse proportion, of the relative amount of water originally present in different parts of the fresh specimen. The experiments reported in the present paper take advantage of this fact, and are based on the assumption that differences in the distribution and concentration of substance, as occurring in cells and tissues find expression, after

* The results reported in this paper were presented at the annual meeting of the Electron Microscopy Society of America, December 11 to 13, 1947, in Philadelphia, Pa.

drying, in variations in height and shape at the surface of the specimens. The results here reported indicate that surface replicas of dried cells and tissues can depict with surprising accuracy morphological details of their internal constitution.

Material and Methods

In the present work the material studied has been single cells, as in blood smears or tissue imprints, and bacterial cells. The preparations were fixed, usually in osmium tetroxide vapors, and allowed to dry in air, or over P_2O_5 .

The technique employed consisted in preparing replicas of the cellular material and recording in the electron microscope the negative image so obtained, a method heretofore used in the study of crystalline structures or metal surfaces (11, 12).

Replicas were obtained by immersing glass slides supporting the dried specimen in a 0.5 per cent solution of formvar E¹ in ethylene dichloride and allowing the preparation to dry while in the horizontal position. The resulting plastic film presented a level surface on its upper side, while retaining the imprint on its under surface of the irregularities reflected at the surface of the specimen. The difficulties encountered in detaching the mold, prior to its transfer to the supporting wire mesh of the electron microscope, varied greatly with the nature, and especially the thickness, of the specimen. The plastic film must be thin to insure contrast in the image made of the replica and its fragility precludes the use of much mechanical force when freeing it from the glass support; hence, conditions must be such that it can be detached readily. This is usually accomplished when the cellular elements projecting into the film, and therefore weakening it in places, are not crowded, but have between them a sustaining network of relatively thick film. Favorable conditions of this sort are provided by using thinly spread blood or bacterial smears, where some free space is left between the cells.

The preparation of the mount, *i.e.* the lifting of the replica from the cells, and its transfer to the wire mesh screen of the electron microscope was carried out under the dissecting microscope. Formvar-coated smears of blood or bacteria were immersed in distilled water and an area, about the size and the shape of the supporting screen, was selected and outlined. This film disc was detached by means of sharpened watchmaker's forceps, and moved over a screen placed alongside beforehand. The screen and the film over it were then lifted from the water and the preparation was drained on blotting paper and allowed to dry. When resorting to shadowing of the replicas, the film was detached, turned over, and spread on a clean glass slide, in the inverted position. The exposed side of the replica was then shadowed in the usual manner.

The observations were made by means of a RCA, type E.M.U., electron microscope.

EXPERIMENTAL

Replicas of Blood Cells.—Fig. 1 shows a micrograph of a replica of a smear of chicken blood. The replica was mounted on the screen of the electron microscope, and the photograph was made with an ordinary microscope, using visible light. The smears had been prepared in the usual manner by spreading thinly on a glass slide a drop of blood obtained from the comb of an apparently normal pullet, fixing it rapidly over osmium tetroxide vapors, and allowing it to dry in air. As shown in Fig. 1, the replica of the smear produces an image

¹ Formvar E (grade No. 15-95), obtained from the Shawinigan Products Corporation, New York.

of the blood cells, so faithful that it is difficult to distinguish, under the light microscope, between the original unstained cells and their plastic mold. As in the direct light microscope examination of the smear itself, the nuclei in the replicas of the erythrocytes are evident, and the leucocyte in the field appears to contain granules. Fig. 2 represents the replica of a chicken erythrocyte, photographed with the electron microscope under a magnification of 2200, and enlarged to 5300. Nearby is what appears to be the "ghost" of a red cell, with remnants of its nucleus. The fact that the body of the apparently intact erythrocyte appears granular, whereas the ghost cell is smooth and homogeneous, is evidence for difference in their surface conditions, or the properties of their membranes. The background of the preparation in Fig. 2 presents a fine granular structure, presumably produced by elements of the plasma.

Fig. 3 shows an electron micrograph of the replica of a mouse leucocyte, apparently a monocyte or a large lymphocyte. About it, can be seen the curved margins of a number of red cells. The replica reveals internal details, notably the shape of the nucleus, and cytoplasmic bodies with the appearance of rod-shaped mitochondria.

Fig. 4 is a micrograph of a chicken platelet, showing the vague outline of what is probably a nucleus, three vacuoles, and small bodies of various shapes which may be mitochondria. Fig. 5 is the replica of a leucocyte of chicken blood. The numerous dark bodies shown in the electron micrograph represent depressions in the surface of the dried cell and probably correspond to what were vacuoles. Differences in the density to the electron beam in these areas may reflect differences in the amount of water which existed originally in the various vacuoles.

Replicas of Bacteria.—Microorganisms are generally surrounded by a voluminous capsule or are encased in a relatively rigid covering. *A priori*, it would seem likely that structural details of their cell content would not be accessible for study by the method of replicas. In fact, it has been possible to obtain replicas, of *E. coli* for the most part, which picture a number of morphological features reflecting, presumably, details of cellular organization of the bacteria.

Figs. 6 to 8 are micrographs of such replicas. Fig. 6 shows a cell with two large bodies, one near the center, and the other near one end. Fig. 7 shows two bacterial cells, one of them appearing but faintly, obviously because it lay partly beneath the other and made but a slight impression on the formvar film. The upper cell shows two terminal bodies, like those of growing organisms, and a central body of smaller size. Fig. 8 represents a portion of a filamentous form of *E. coli*, such as develops in aging cultures. The white bodies which appear in the organism seem to be arranged along a spiral path extending from one to the other end of the filament. The same bodies have been noted in replicas of similar elongated organisms. They appear to be sufficiently

rigid to cause the cell wall to rupture over them, probably during desiccation; in the picture this is noticeable at both ends of the filament. The nature of inclusions of identical morphology detected with the ordinary microscope in specimens of various forms of *E. coli* is not known; the fact that they stain with methyl green might be taken to indicate that they contain in appreciable amounts substances related to chromatin and that they represent bacterial nuclei.

DISCUSSION

Replicas of the surface of certain cells have occasionally been obtained and photographed in the electron microscope (13, 14) but it seems that full advantage has not been taken of this interesting technique, and the fact that it can furnish information concerning the internal structure of cells has not been realized. These have been the objects of the work reported in the present paper. Replicas of blood cells and bacteria have been obtained which not only give the shape of the cells but show nuclear membranes and what appears to be chromatin structures, mitochondria, and vacuoles. An important feature of the method is that the thickness of the specimen, often a limiting factor in the electron microscopy of cells, may no longer be significant if replicas of the proper thinness can be prepared. The method would appear useful in the study of erythrocytes and bacteria, and in the case of the nuclear region, which remains too thick for direct electron microscopy even in thinly extended cells in tissue cultures. That the method of replicas, as applied to the study of cells, can be technically improved is probable, so that higher resolution may be obtained and even finer details of internal structure may be revealed. The hope seems warranted that the method may assist in the study of problems to which direct electron microscopy cannot yet be applied, as in the case of the intracellular growth of malarial parasites, the penetration and growth of bacterial viruses, and the morphology of chromosomes.

SUMMARY

The method of replicas has been applied to the study with the electron microscope of blood cells and bacteria.

The results indicate that the method can reveal details of intracellular structures. Nuclei can be perceived, and also cytoplasmic bodies such as mitochondria and vacuoles.

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EXPLANATION OF PLATES

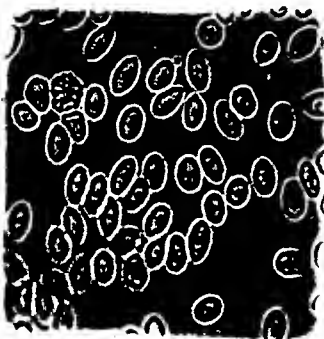
All preparations illustrated in Figs. 1 to 8 were fixed by exposure to osmium tetroxide vapors for 5 to 15 minutes, and subsequently were allowed to dry in air. Since the pictures were made from replicas, all illustrations are negative images of the original cells.

PLATE 16

FIG. 1. Formvar replica of a smear of chicken blood. The imprints left on the plastic film reproduce to a striking extent the appearance of the original blood cells as viewed without staining. The picture shows many erythrocytes, and one leucocyte. Photograph made with an ordinary microscope and visible light at a magnification of 250, enlarged to 450.

FIG. 2. Formvar replica of chicken blood cells. On the left, is an apparently intact erythrocyte; on the right, what would appear to be the ghost of a red cell. Electron micrograph taken at a magnification of 2200, and enlarged to 5300.

FIG. 3. Replica of mouse leucocyte, probably a monocyte, or a large lymphocyte. The elongated bodies in the cytoplasm correspond to mitochondria. Electron micrograph taken at a magnification of 2600, and enlarged to 4000.



1



3



2

PLATE 17

FIG. 4. Replica of a chicken platelet. A nucleus is faintly outlined. The dark cytoplasmic bodies presumably correspond to vacuoles, the light bodies probably to mitochondria, judging from the bulges and depressions, respectively, that they left on the replica. Electron micrograph taken at a magnification of 2600, and enlarged to 5200.

FIG. 5. Replica of a chicken leucocyte. The areas free of granules correspond to two lobes of the polymorphic nucleus. Electron micrograph taken at a magnification of 2200, and enlarged to 4400.

FIG. 6. Replica of an organism from an *E. coli* culture, with two relatively large internal bodies, one near the middle of the cell, the other near one end. Electron micrograph taken at a magnification of 2600, and enlarged to 7800.

FIG. 7. Replica of organisms from an *E. coli* culture, showing one cell lying partly over another. The upper cell presumably made the more effective replica. It has one rounded body at each end, and a central one of smaller size. Electron micrograph taken at a magnification of 2600, and enlarged to 7800.

FIG. 8. Replica of a filamentous organism, of a type frequently found in "old" cultures of *E. coli*. The picture shows discrete bodies, apparently arranged along a spiral path. In ordinary microscopic preparations, methyl green stains bodies similar to those shown, and also to the larger ones of Figs. 6 and 7. Electron micrograph taken at a magnification of 2600, and enlarged to 5200.



4



5



6



8



7

AN ELECTRON MICROSCOPE STUDY OF SALIVARY GLAND CHROMOSOMES BY THE REPLICA METHOD

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PLATES 18 TO 20

(Received for publication, December 15, 1948)

Information concerning the structure of the giant chromosomes of dipteran salivary glands has derived mainly from the application of cytological procedures, histochemical techniques, and micromanipulation (1), methods which have utilized the limited resolving power of the light microscope. No studies have been recorded in which the increased resolution afforded by the electron microscope has been used to elucidate the organization of the giant chromosomes, although a number of attempts have been made to study the chromosomes of plants (2, 3), mammals (4), and birds (5) by means of this instrument. Such attempts have been impeded by the thickness of the chromosomes and their opacity to the electron beam; hence little or no fine structure has been revealed. In the present investigation this technical difficulty was obviated by resorting to a modification of the simple replica method (6) used previously in the study of metal surfaces. Replicas of salivary gland chromosomes obtained by this method reveal considerable detail not apparent in electron micrographs of the original chromosomes.

Material and Method

Preparation of Chromosomes.—Full grown larvae of *Drosophila pseudoobscura* and *Drosophila melanogaster* Setw* and Ore R, cultured on corn meal-molasses-agar, were used for this study.[†] The larvae were placed in a drop of 45 per cent acetic acid and the salivary glands were isolated under the dissecting microscope by means of stainless steel needles. Adherent fat body was discarded. Only those glands filled with secretion and composed of large, flat cells were selected for study. The large-cell tip of the gland was then transferred to a fresh drop of 45 per cent acetic acid on a glass slide usually coated beforehand with a thin film of Mayer's albumen. A coverslip was laid over the preparation, the slide was inverted onto a paper towel, and the cells were crushed by the application of pressure through the slide. By this means the tissue was spread out very thin and flat so that Lieberkühn's rings appeared. Examination under the light microscope showed that in successful preparations practically all the cells had been disrupted, with the chromosomes dispersed, individually extended, or clustered out, many of them isolated and apparently free from nuclear and cytoplasmic debris. For further fixation and protective dehydration these preparations were exposed to

* This work was supported by the Medical Sciences of the National Research Council.

[†] The *Drosophila* cultures of these larvae were kindly provided by Mr. Bruce Wallace of the Cornell Institution of Washington, Cold Spring Harbor, New York, for whose generous contribution the authors are grateful.

alcohol vapors for 12 to 24 hours by keeping them in a closed Coplin jar containing just enough 95 per cent alcohol to reach the lower edges of the coverslips. The preparations were finally immersed in 95 per cent ethyl alcohol for at least 24 hours.

Preparation of Replicas.—The coverslip was carefully pried loose from the slide while still immersed in 95 per cent alcohol. The slide and its separated coverslip were each passed through three changes of fresh absolute ethyl alcohol and placed in a desiccator over phosphorus pentoxide until all visible moisture had vanished.

In many preparations the chromosomes remained adherent to the coverslip rather than to the slide after they had been separated. When the coverslip was used in making replicas it was first cemented with clarite to a glass slide, tissue side up.

Replicas were made by dipping the slide vertically into a 0.75 per cent solution of formvar² in ethylene dichloride just long enough to immerse and withdraw. Upon removal the slide was kept in the vertical position while the excess formvar was drained off, the back of the slide was wiped with a gauze pad, and the film was allowed to dry in air. When the humidity was high it was necessary to dry the film in a phosphorus pentoxide desiccator, because the cooling effect of the rapidly evaporating ethylene dichloride would cause water to condense on the film, which would then become opaque and brittle.

Stripping of the film from the slide was carried out in water under the dissecting microscope by means of sharpened watchmaker's forceps. In regions where the chromosomes appeared properly spread out and numerous, circular areas were outlined and discs of the film, just large enough to cover the specimen screen of the electron microscope, were peeled off and brought to the surface of the water where they immediately flattened out, and picked up on 160 mesh stainless steel screens. The screens with their films were drained by touching them to a lintless towel and were allowed to dry in air. Replicas thus obtained were then ready for examination in the electron microscope. The whole procedure of coating the slide with formvar and mounting the film on the specimen screen took about 15 minutes.

Chromosome preparations from which replicas have been obtained can be used repeatedly for the same purpose if the slide is freed of remaining formvar by stripping and then dried after passage through several changes of absolute alcohol.

All micrographs were made with the RCA (type E.M.U.) electron microscope.*

Comments on the Method.—Dehydration and complete drying of the spreads were found necessary for successful preparation of replicas. If the material dried from an aqueous medium, the chromosomes shrank considerably and were distorted. If the spread was not allowed to dry in the air, but was passed from absolute alcohol through ethylene dichloride into the formvar solution, the chromosomes became embedded in the film and came off the slide with it. Even when the slide had been dried in air, the chromosomes would come off with the film if the slide was allowed to stay in the formvar solution too long. It is necessary to withdraw the slide as soon as it has been immersed.

The thickness of the chromosomes is another important factor in making replicas. Good replicas could be made only when the dried chromosomes were sufficiently thin, as indicated by their transparency and lack of distortion when examined with the high dry power of the light microscope ($\times 600$). Such chromosomes were almost invisible unless examined by diffused or diminished light. The thickness of these preparations depended upon the pressure applied in crushing the salivary glands and the length of time they were held between slide and coverslip in the alcohol vapor. If the preparation was too thick the chromosomes shrank

² Formvar E (grade No. 15-95), obtained from the Shawinigan Products Corporation, New York.

* Made available through the courtesy of Dr. R. M. Taylor, Director of the laboratories of the International Health Division of The Rockefeller Foundation.

in drying and appeared opaque and distorted. Thus the appearance of the dried chromosomes under the light microscope indicated whether they were suitable for the preparation of replicas.

A certain thickness of the formvar film was also found to be essential for the success of the method. Films that were too thin were not only difficult to strip but also gave no detail. Films that were too thick could be detached readily but gave poor contrast in the electron microscope and exhibited many defects, such as tears and holes in the replicas. Whether the film was of optimal thickness could be readily ascertained while it was still on the slide immersed in water. Under favorable conditions numerous tiny water droplets penetrate under the film, causing minute elevations which have a golden color when viewed under the dissecting microscope. Such a film can be easily detached from the slide and when placed on a screen and dried has a golden color in daylight (angle of incidence approximately 80°). According to Schaefer and Harker (6), this color is given by a film 70 m μ in thickness, but measurements of our films with polarized light⁴ revealed an average thickness in the area of the replicas of about 130 m μ .

Care had to be taken that the slides and coverslips were free from all traces of grease, for this prevents stripping of the plastic film. Only new slides and coverslips were used, cleaned in sulfuric acid-bichromate solution and dried from alcohol.

Numerous attempts were made to change the fixation of the chromosomes in order to improve the definition of the replicas. Osmium tetroxide, trichloroacetic acid, phosphotungstic acid, and Flemming's mixture were all tried in various combinations, but without success. Either the chromosomes were not preserved or replicas could not be made from them.

Experimental.—In an attempt to localize desoxyribonucleic acid in the chromosomes, dried squash preparations of salivary glands were subjected to digestion by desoxyribonuclease (7) in a mixture containing, as final concentrations, 0.01 mg. enzyme⁵ per ml., 0.025 M phosphate buffer of pH 7.3, and 0.005 M magnesium sulfate. Control mixtures were identical except that no desoxyribonuclease was added.

The salt mixtures were prepared on the day before the experiment and were kept at 37°C . overnight. The dry enzyme was dissolved in the warm salt mixture just before use. Both the digesting and control mixtures had a final pH of 7.45. The preparations were incubated at 37° for 60 minutes, washed in five changes of distilled water, passed through three changes of fresh absolute alcohol, and dried in air for 3 days. Formvar replicas of the chromosomes were then made in the usual manner. After the replicas had been taken, both controls and the digested preparations were treated according to the Feulgen nuclear technique.

OBSERVATIONS

As the replicas described in this report are, in effect, casts of the original chromosomes, the electron micrographs obtained from them are negative images. Therefore, in the final prints the thinner portions of the chromosomes are represented by dark grey or black, and the thicker portions by light grey or white.

Because the replicas were surface impressions it was essential that the chromosomes be separated from one another and be free of overlying debris which would obscure structural details. For this reason the replicas studied

⁴Work supported in part by the U. S. Office of Naval Research, The Rockefeller Institute for Medical Research, and the National Cancer Institute.

⁵The source of partially purified desoxyribonuclease was kindly supplied by Dr. M. S. Brown of the Department of The Rockefeller Institute for Medical Research.

were selected from those preparations in which the chromosomes appeared untangled and cleanly separated from cellular debris. Even so, it was not possible to obtain a complete, satisfactory record of a whole chromosome, because twists and turns sometimes distorted considerable portions of it and because the thicker bands often revealed little or no detail. Since moderately stretched chromosomes of *Drosophila*, exclusive of the short fourth chromosome, are 220 to 485 μ in length (8), an entirely uncoiled chromosome would extend across several squares of the specimen screen, and appreciable portions would be hidden by the intervening wires of the screen. Hence a complete picture of an uncoiled chromosome could not be made.

As can be seen from an examination of Figs. 1, 2, and 3, the giant salivary gland chromosome in the unstretched condition appears to consist of a series of closely apposed rows or bands of small round bodies extending across its width. In the clearest band in Fig. 1, a row of bodies can be discerned measuring from 250 to 330 $m\mu$ in diameter. Between granules and between rows there are no obvious connections. The discreteness of the bodies can be seen in Fig. 2 where the chromosome is slightly stretched. In Fig. 3 the striated and granular structure is also evident, with no indication of strands or threads between granules or bands. In all the micrographs these granules have been measured wherever their contours were clearly discernible. They were found to vary from 210 to 330 $m\mu$ in diameter, the majority being in the range of 250 to 290 $m\mu$. Since these granules are therefore just at the limit of resolution of the light microscope, they are considered to be identical with the small basophilic granules and vesicles that have been designated as chromomeres (1).

The micrographs of the replicas reveal no evidence of a limiting membrane surrounding the chromosomes, either in lax or stretched specimens. The chromomeres extend to the edge of the chromosome, and no sheath or pellicle appears to intervene between the outermost chromomeres in a band and the material around the chromosome (Figs. 1, 3, and 4). A definite matrix of intrachromosomal substance between chromomeres also appears to be absent.

When chromosomes are moderately or greatly stretched, the bands of chromomeres separate, and more or less coarse longitudinal strands appear between them (Figs. 4, 5, and 6). In some places these filaments stretch between corresponding granules of neighboring bands as at *a* in Fig. 4; in other places (*b* in Figs. 4 and 5) they form an interweaving longitudinal meshwork. Nowhere could the same strand be clearly traced across more than one row of granules. The replica method has not revealed any fine periodic structure in the strands such as has been found in myofibrils or collagen threads (9). They seem, on the contrary, to be homogeneous fibers which divide and anastomose between bands, their configuration depending upon the amount and direction of the stresses developed in stretching the chromosomes (Fig. 6).

The digestion experiments with desoxyribonuclease provided further infor-

mation about the strands. Figs. 7 and 8 show portions of moderately and greatly stretched chromosomes after they had been subjected to the action of desoxyribonuclease for 60 minutes at 37°C. These chromosomes give a negative Feulgen reaction when tested after the replicas had been made, thus indicating that the desoxyribonucleic acid had been removed by the digestion. The continuity of the chromosomes was not affected by the procedure but the chromomeres appeared to be more discrete than those in untreated preparations. In the heavier bands small granules can be discerned that are not obvious in such bands of undigested chromosomes (compare Fig. 7 with Fig. 3). The distinct chromomeres, measuring from 210 to 250 $m\mu$ in diameter, were also a little smaller than those of the control chromosomes. Between the bands no fibers or filaments remained, but in their place was an amorphous material that had no apparent orientation in relation to the chromomeres. The control preparations resembled in all respects the untreated chromosomes (compare Fig. 8 with Figs. 4 and 5).

DISCUSSION

The replica method has already been applied to blood cells and bacteria with some success (10), and it seems probable that with certain modifications it can be used to study tissues which have thus far proved inaccessible to electron microscopy because of their opacity to the electron beam. In the present study on giant salivary gland chromosomes by means of the replica technique, no detail has been revealed that had not been suggested by examination of stained preparations with the light microscope. However, the increased resolution and magnification provided by the electron microscope have made it possible to characterize the structure of these chromosomes more fully.

According to the most widely accepted view, the giant chromosomes consist of a number of parallel and closely approximated threads or chromonemata bearing homologous chromomeres at regular intervals marked by Feulgen-positive, basophilic bands. This polytene structure was postulated by Koltzoff (11) and Bridges (8) on the basis of fixed and stained preparations and has been supported by the work of Bauer (12), Painter and Griffen (13), and d'Angelo (14). Metz and Lawrence (15) have presented contrary evidence to the effect that the chromosomes are alveolar in structure, made up of achromatic vesicles or droplets in a chromatic matrix, and that the strands visible in stretched chromosomes are not true chromonemata but artifacts, stress lines in the matrix produced by the stretching of chromatic material from the band regions. Budd's ultrastructural studies (16) on fixed salivary gland chromosomes have tended to confirm this interpretation. The polytene theory has also been challenged by Ris and Crouse (17), who state that the bands are actually caused by the complex coiling of a bundle of chromonemata which weave back

and forth across the width of the chromosome. According to this view the chromonema is uniformly Feulgen-positive and the so called chromomeres are optical sections of gyres in the chromonema. The electron micrographs presented in this paper do not substantiate any one of these theories.

Although there is general agreement (1) that a sheath envelops the chromosomes, we could find no evidence for it in the replicas. Chromomeres occupy the entire width of the chromosomes. It is possible that the sheath may be too delicate to withstand squashing of the chromosomes, but the fact that the nuclear membranes of blood cells can be demonstrated in replicas (10) makes it seem improbable that the replica technique would not disclose a sheath in the chromosomes if it were present. However, in view of the apparently conclusive micrographical demonstration by d'Angelo (14) of the existence of a membrane in the living giant chromosomes of *Chironomus*, it may be inferred that in our material the sheath was destroyed or dispersed by the process of making squash preparations.

The existence and character of a chromosomal matrix in which the chromonemata and chromomeres are embedded have been controversial subjects for many years (1). The observations reported here provide no evidence for the presence of any intrachromosomal substance distinct from the chromomeres. Neighboring rows of chromomeres lie in very close apposition in the unstretched chromosomes. The appearance of achromatic interband regions in stained lax chromosomes may be merely the result of the relatively poor nucleic acid content of certain bands.

The nature of the strands seen in electron micrographs of the interband regions of stretched chromosomes is more difficult to determine. The facts that these filaments are not apparent in the lax chromosomes, that they are evident only in stretched preparations, that they divide and anastomose between bands, and that they disappear when digested by desoxyribonuclease suggest that they are artifacts, as Metz and his coworkers maintain. Some indication of their nature can be obtained by comparing Fig. 5 in the present paper with the electron micrographs of thymonucleohistone in the paper of Mazia, Hayashi, and Yudowitch (18). These workers compressed films of thymonucleoprotein into fibers which under the electron microscope appear as narrow, thin, anastomosing sheets, resembling the strands that form between bands of stretched chromosomes. This similarity is further borne out by the results of our digestion experiments with desoxyribonuclease. These results suggest that when the chromosome is stretched desoxyribonucleoprotein is pulled out from the surface of adjoining chromomeres into sheets extending between them and that these sheets tear or shred into strands according to the irregular stresses produced by pressure on the coverslip and the varying resistances of the bands. When nucleic acid is removed by enzyme action, the strand-like appearance is destroyed and only the protein remains as amor-

phous material. This interpretation of the strands, furthermore, is in accord with the fact that the interband regions of salivary gland chromosomes are positively birefringent to polarized light only when in the stretched condition (19).

The electron micrographs show that the transverse bands are indeed composed of small round granules arranged in rows across the width of the chromosomes. Since the replicas are surface impressions there can be no possibility of confusion arising from misinterpretation of optical sections. It is difficult to visualize how the small round bodies seen in these preparations could be caused by gyres of a continuous uniform thread. Moreover, the persistence of these bodies after the chromosome has been subjected to digestion by desoxyribonuclease indicates that they have a complex constitution. They contain at least two types of substances, desoxyribonucleic acid and other constituents, probably protein in nature. Since the granules are smaller after digestion and since strands that are destroyed by desoxyribonuclease appear between them when the chromosome is stretched, at least part of the desoxyribonucleic acid lies on the surface. The fact that after digestion granules are more readily discerned in the heavy bands also supports this inference. Evidently the nucleic acid component does not determine the shape of the chromomeres.

In general the observations reported here lead to the concept that the giant salivary gland chromosome is essentially chromomeric in structure. The chromomeres lie in close apposition to one another to form continuous, parallel, adherent chains without interpolated threads. Each member of a chromosome pair is, then, a single giant chromonema composed of chromomeres that are more numerous, or perhaps larger, or both, than those in the chromosomes of ordinary cells.

SUMMARY

A method for preparing replicas of salivary gland chromosomes for electron microscopy is described.

Electron micrographs of these replicas show that the giant chromosomes are composed of a series of small granules of approximately equal size arranged transversely across the chromosome.

In stretched preparations a linear network of filaments appears between the rows of granules. These fibers cannot be traced between corresponding granules of more than two consecutive rows. When the chromosomes are digested by desoxyribonuclease, these fibers disappear and only amorphous material remains between the bands. The characteristics of the strands suggest that they are artifacts produced when the chromosomes are stretched.

The small granules are composed of desoxyribonucleic acid and at least one other component, probably a protein. The nucleic acid seems to lie at least in part on the surface of each granule.

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EXPLANATION OF PLATES

All figures are electron micrographs of formvar replicas made from giant salivary gland chromosomes fixed in 45 per cent acetic acid and dehydrated and dried from absolute ethyl alcohol. The thicker portions of the chromosomes are represented by light grey or white, the thinner portions by dark grey or black.

PLATE 18

FIG. 1. Portion of an unstretched chromosome of *D. melanogaster* Sc^{8w}. The rows of granules (chromomeres) are close together, with no apparent interconnecting threads. Micrograph taken at a magnification of 2200, enlarged to 8140.

FIG. 2. Part of a slightly stretched chromosome of *D. melanogaster* Sc^{8w}, showing rows of chromomeres separated by short distances. Fiber formation is restricted to the lower edge of the chromosome. The thin, diagonal, dark line (arrow) extending along the length of the chromosome marks the groove between the two component chromatids coiled loosely about each other. Micrograph taken at a magnification of 2200 enlarged to 8140.

FIG. 3. Portion of a chromosome of *D. melanogaster* Sc^{8w}, showing chromomeric structure and coiling of the chromatids. The arrow points to the groove between chromatids. Micrograph taken at a magnification of 2600, enlarged to 9620.

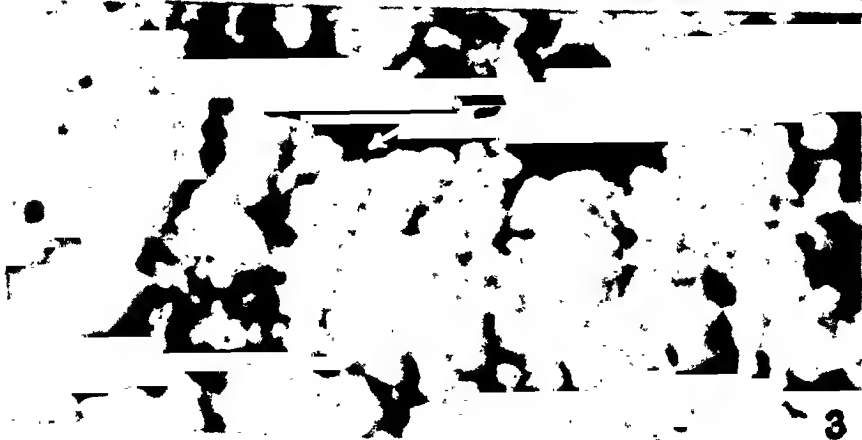


PLATE 19

FIG. 4. Portion of a stretched chromosome of *D. melanogaster* Sc^sw^a, showing at *a* the filaments extending between corresponding chromomeres of neighboring rows. At *b* the filaments are interconnected to form a meshwork. Stretching of the chromosome has somewhat separated the round bodies or granules from one another. Micrograph taken at a magnification of 2200, enlarged to 8140.

FIG. 5. Portion of a greatly stretched chromosome of *D. melanogaster* Ore R, showing meshwork of filaments between bands of chromomeres (*b*). Micrograph taken at a magnification of 2600, enlarged to 9620.

FIG. 6. Portion of a greatly stretched chromosome of *D. melanogaster* Sc^sw^a, showing two bands composed of granules approximately 240 m μ in diameter, connected by longitudinal strands. Micrograph taken at a magnification of 3100, enlarged to 9455.

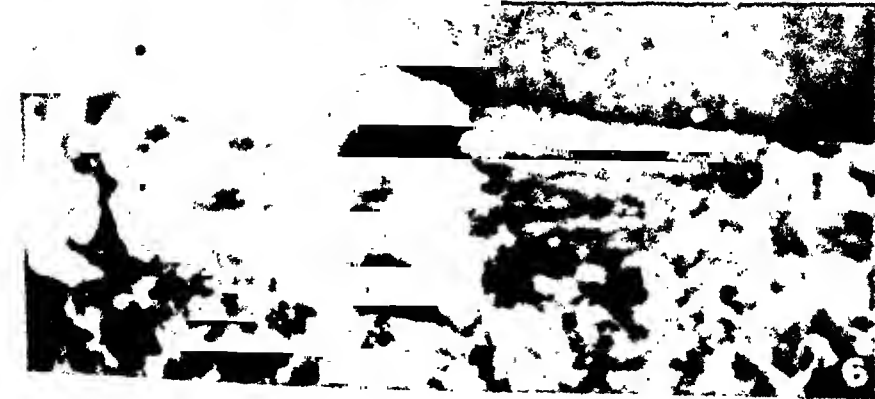
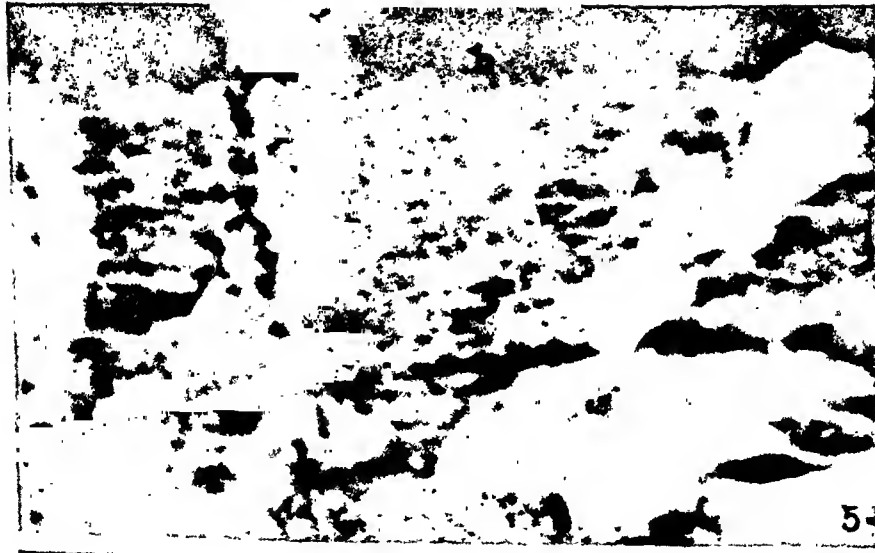
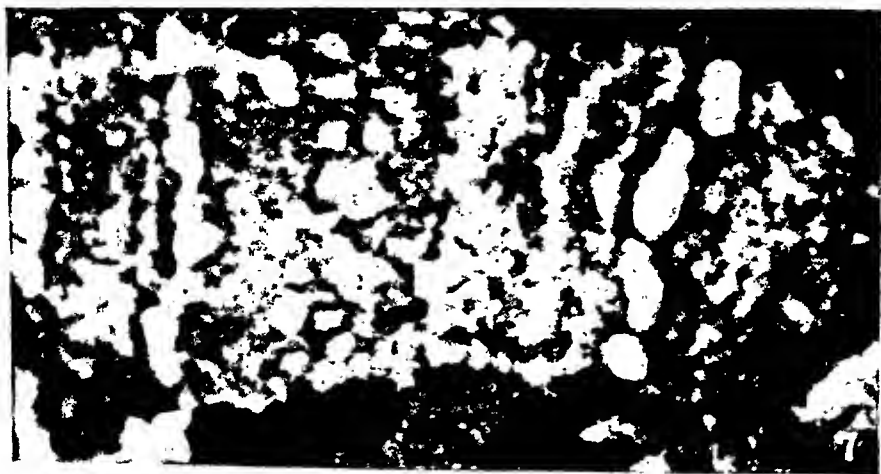


PLATE 20

FIGS. 7 and 8. Portions of moderately and greatly stretched chromosomes of *D. melanogaster* Ore R after digestion with desoxyribonuclease for 60 minutes at 37°C. The chromosomes from which the replicas were made were Feulgen-negative. The chromomeres appear to be smaller and more distinct than those of undigested preparations. No filaments remain after digestion, but in their place an amorphous coagulum appears between the bands. Electron micrograph taken at a magnification of 3100, enlarged to 9455.



A TYPE-SPECIFIC PROTEIN FROM PNEUMOCOCCUS*

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Though the presence of protein antigens in pneumococcus has long been recognized, type specificity within this species has been defined by capsular carbohydrates. Much of the work on protein antigens of pneumococcus has dealt with a nucleoprotein fraction described by Avery and his coworkers (1), and there is general agreement that this material exhibits species specificity. In quantitative studies of pneumococcus R-anti-R systems, however, Heidelberger and Kabat (2) obtained results indicating the existence of a minor type-specific protein antigen in a rough variant of pneumococcus type II (D39R), and later Heidelberger (3) suggested that the results permitted envisaging the possible existence, in non-encapsulated pneumococci, of an antigen that possesses type specificity.

In the hemolytic streptococci of Lancefield group A, in contradistinction to pneumococcus, protein antigens play a primary rôle in the determination of type specificity (4). Of these protein antigens, the M substance isolated by Lancefield, which belongs to the class of alcohol-soluble proteins, is of especial importance. Proteins of this class have been found in several bacterial genera (5) and their presence in pneumococcus has been noted heretofore (6). The present studies are concerned with the properties and type specificity of such alcohol-soluble M proteins of pneumococcus and with the demonstration of their marked similarity to the M proteins of group A streptococci.

cus type XIX horse serum prepared by the New York City Department of Health and possessing a high titer of anti-C carbohydrate antibody was also employed.

Preparation of M Protein Extracts.—Extracts of pneumococci were made according to the method of Swift, Wilson, and Lancefield (8). To obtain a heavy growth of organisms for extraction, 1 per cent glucose was added to neopeptone broth cultures of pneumococci after incubation overnight and the acid formed on subsequent incubation was neutralized intermittently with 3 N NaOH. The organisms were collected by centrifugation in the cold in an angle centrifuge prior to extraction. To the packed cells, sufficient N/5 HCl was added to adjust the pH to 2.0-2.4. The acid suspension was heated in a boiling water bath for 15 minutes, cooled, centrifuged, and the supernatant decanted. To the clear supernatant, a drop of 0.01 per cent phenol red was added and the solution was adjusted to neutrality with N/2 NaOH. The cloudy solution was centrifuged again and the resultant clear supernatant was used for testing. The extract thus obtained contains the M protein, the somatic or C carbohydrate, and if an encapsulated organism is employed, the capsular carbohydrate as well.

Partial Purification of M Protein.—Two methods were employed to separate the M protein from C carbohydrate present in the cell extracts described. The addition of 1 to 2 volumes of 95 per cent ethyl alcohol to the neutralized extract precipitated the M protein and left most of the somatic carbohydrate in solution. This could be precipitated by the addition of 4 to 5 volumes of alcohol. The precipitates were collected by centrifugation and were readily soluble in normal saline.

The M protein was purified partially also by the picrate method that Dodds and Dickens (9) employed for the purification of insulin. To 2 volumes of cell extract was added 1 volume of saturated picric acid solution. The precipitate was collected by centrifugation and extracted with 2 volumes of 70 per cent aqueous acetone. To the acetone extract were added an equal volume of water and saturated picric acid. The second precipitate was collected and treated with 1 to 2 volumes of acid alcohol (95 per cent C_2H_5OH , 3 volumes, 3 N HCl, 1 volume). The acid alcohol extract was added to 20 volumes of cold acetone from which the hydrochloride of the M protein precipitated in flocculent particles after several hours. The precipitate was collected by centrifugation and was readily soluble in normal saline.

Precipitin and Agglutination Tests.—Precipitin tests were carried out either in small test tubes or in capillary tubes according to the method of Swift, Wilson, and Lancefield (8). With the test tube technique, 0.3 cc. of varying dilutions of cell extract was layered over 0.3 cc. of diluted serum containing 2 parts of serum and 3 parts of normal saline. The tubes were incubated for 1 hour at 37°C. and the reactions read after standing overnight in the ice box. With the capillary method, undiluted serum was used and the reactions read after standing for 16 hours at room temperature. Agglutination tests were carried out with a constant amount of vaccine suspended in normal saline or in saline buffered with M/50 phosphate buffer pH 7.2 and with serial dilutions of serum. Tests were read after 1 hour's incubation at 37°C. and overnight incubation at 4°C.

Strains of Pneumococcus.—In the work reported in this paper and in the following one (10), the naming of the colonial variants of pneumococcus employed is attended with certain difficulties. Two nomenclatures for the colonial variants of pneumococcus are already extant. That of Griffith (11) includes only the encapsulated form designated by the letter S (smooth) and the more widely known of the unencapsulated forms designated by the letter R (rough), selected by growing encapsulated cells in antiserum directed against the capsular polysaccharide. This terminology has enjoyed general use in the description of pneumococcal strains. In 1934, Dawson (12) reported the existence of a second unencapsulated variant of pneumococcus, and to bring the nomenclature of pneumococcal colonial forms into conformity with that of other bacterial species, he suggested that encapsulated forms be designated by the letter M (mucoid), the previously described unencapsulated form by the letter S (smooth), and the newly described unencapsulated form by the letter R (rough) by virtue of its distinctive colon-

ial morphology and autoagglutinable properties. There have been few reports concerning all three colonial variants of pneumococcus and the terminology of Dawson has not been used widely. It is unfortunate that the nomenclatures of Griffith and of Dawson employ the same symbols to designate different colonial forms, for inevitable confusion is created thereby. In the strains to be reported, variants designated smooth by Griffith and mucoid by Dawson will be referred to as encapsulated forms. The more generally recognized unencapsulated variant known as rough in Griffith's nomenclature and smooth in Dawson's terminology, will be designated by the term Griffith rough, and the less widely studied unencapsulated variant described first by Dawson will be described by the term Dawson rough. This terminology, while admittedly unsatisfactory, should minimize ambiguity. A more satisfactory nomenclature awaits a better understanding of colonial variation. Appended below is a list of the strains of pneumococci used in the present studies. Encapsulated strains are grouped by capsular type and unencapsulated strains are described immediately after the parent strain.

Capsular Type I

- I-SV1: an encapsulated strain carried for many years in the laboratory.
- I-SV1-P26: an encapsulated, sulfonamide-resistant strain derived from I-SV1 in 1938.
- I-192R: a Griffith rough strain derived from I-SV1 and carried for many years in the laboratory.
- I-192RD: a Dawson rough strain derived from I-192R in 1948.
- I-Rack: an encapsulated strain carried for several years in the laboratory.
- I-Moody: an encapsulated strain carried for several years in the laboratory.
- I-JHHA: an encapsulated strain recovered from a brain abscess in Baltimore in 1948.
- I-JHHAR: a Griffith rough variant derived from strain I-JHHA.
- I-JHHB: an encapsulated strain isolated from a patient with lobar pneumonia in Baltimore in 1948.

Capsular Type II

- II-D39S: an encapsulated strain carried for many years in the laboratory.
- II-R36A, II-R36T, and II-R36NC: three colonial variants of the Griffith rough strain II-R36 derived from II-D39S.
- II-R36ND: a Dawson rough variant derived from II-R36NC in 1948.
- II-BW: an encapsulated strain isolated from a patient with lobar pneumonia in New York in 1948.
- II-BWR: a Griffith rough variant derived from strain II-BW.
- II-BG and II-BS: two encapsulated strains isolated from patients with lobar pneumonia in New York in 1948.
- II-Rat: a strain isolated from a rat during an epizootic in Baltimore in 1947.
- II-RatR: a Griffith rough variant derived from strain II-Rat.
- II-JHH: an encapsulated strain isolated from a patient with lobar pneumonia in Baltimore in 1948.

Capsular Type III

- III-46 and III-SV3: encapsulated strains carried for many years in the laboratory.
- III-46R: a Griffith rough variant of strain III-46.
- III-L and III-H: two encapsulated strains isolated from throat cultures in New York in 1948.
- III-L-1: an intermediate encapsulated strain carried for several years in the laboratory.

Capsular Type VIII

VIII-H: an encapsulated strain carried for several years in the laboratory.

VIII-R13: a Griffith rough variant derived from strain VIII-H.

VIII-RIH: an encapsulated strain carried for several years in the laboratory.

VIII-Romano: an encapsulated strain isolated from a patient with lobar pneumonia in New York in 1948.

VIII-Marino: an encapsulated strain isolated from a patient with meningitis in New York in 1948.

Capsular Types IV, V, VII, IX, XA, XIV, XVII, XIX, XXIVA

All these strains are strains of encapsulated organisms carried in the laboratory for several years.

EXPERIMENTAL

Type Specificity of Anti-M Protein Sera.—Sera were prepared by immunizing rabbits by the method described in the preceding section with heat-killed vaccines of Griffith rough pneumococcal strains I-192R, I-JHHAR, II-R36T, II-BWR, III-A66R2, and VIII-R13 derived respectively from pneumococci of capsular types I, I, II, II, III, and VIII. These sera, when tested before absorption with extracts of homologous and heterologous pneumococci, gave heavy precipitates with the homologous extracts and also of varying but lesser amount with heterologous extracts. By absorbing the sera with the mass growth of one or more strains of heterologous Griffith rough organisms, it was possible to remove the anti-C carbohydrate and other species-specific antibodies and also cross-reacting anti-M protein antibodies. In addition, to insure the absence of antibody against the capsular carbohydrate of the parent strain, the sera were absorbed with 0.1 volume of a 1:10,000 solution of capsular carbohydrate. Such absorbed sera, when tested by either the capillary or test tube precipitin technique, reacted specifically with cell extracts of the homologous strain (Table I). The type-specific designations of the M proteins studied are recorded in Arabic numerals below the names of the pneumococcal strains employed in making anti-M sera. Subsequent reference to anti-M sera will be to sera absorbed with the strains listed in column 2 of Table I unless otherwise noted.

Three additional facts of interest are recorded in Table I. Although strains I-192R and I-JHHAR were both derived from organisms of capsular type I, they possess different M proteins. A similar difference in antigenic composition is manifested by the two strains II-R36T and II-BWR both derived from organisms of capsular type II. In addition, there is recorded a cross-reaction between the sera and extracts of strains I-JHHAR and II-BWR. When the two sera are absorbed reciprocally, precipitating antibodies for both homologous and heterologous strains are removed. It appears, therefore, that organisms of the same capsular type may be associated with different M proteins and that

organisms of different capsular type may possess the same or closely similar proteins. There is shown also in Table I the absence of serological relationship between the M proteins of pneumococci of capsular types III and VIII. This finding is of interest in view of the known immunological relationship of the capsular polysaccharides of these two types.

TABLE I

Type Specificity of Pneumococcus Anti-M Protein Sera as Demonstrated by Capillary Precipitin Tests

Antiserum prepared against strain	Antiserum absorbed with strains	Extract						Pneumococcus C carbohydrate 1:50,000	Homologous capsular SSS 1:50,000	Saline control
		I-192R	I-JHIIAR	II-R36A	II-BWR	III-A66R2	VIII-R13			
I-192R Type 1M	III-A66R2 VIII-R13	++++	-	-	-	-	-	-	-	-
I-JHIIAR Type 2M	I-192R II-R36A VIII-R13	-	++	-	++	-	-	-	-	-
II-R36T Type 2'M	I-192R III-A66R2 VIII-R13	-	-	++++	-	-	-	-	-	-
II-BWR Type 2M	II-R36T III-A66R2 VIII-R13	-	++	-	++	-	-	-	-	-
III-A66R2 Type 3M	I-192R II-R36A	-	-	-	-	++++	-	-	-	-
VIII-R13 Type 5M	I-192R	-	-	-	-	-	++	-	-	-

In Table II are shown the reactions of the sera listed above with extracts of 34 pneumococcal strains including 13 capsular types and their derivatives. All the strains of capsular types III and VIII examined reacted specifically with antisera prepared against a Griffith rough variant derived from the laboratory strain of the homologous capsular type. Strains of capsular type I and II, on the other hand, showed heterogeneity of their M protein component. Two different proteins were found associated with each of these capsular types and in addition, strains of both types I and II were encountered that failed to react with antisera against any of these proteins.

Effect of M Protein on Colonial Variation.—The relation of M protein to colonial variation is shown also in Table II. This type-specific component

was present in the cells of both encapsulated and Griffith rough colonial variants. Its presence in quantity in the latter colonial form distinguishes it from the M protein of group A β -hemolytic streptococci, for little or no type-specific protein is found in the glossy variant of streptococcus which has been considered to be the colonial analogue of the Griffith rough pneumococcus (13). Further degraded rough colonial variants of pneumococcal strains I-192R and II-R36NC, obtained by the method of Dawson, were studied also for the presence of M protein. Although the component could not be detected occasionally in Dawson rough variants derived from pneumococcus I-192R, consistently deficient cultures were not obtained.

Capillary Precipitin Reaction

Antiserum (absorbed as in Table I)	I 192R	I SV1 P86	I 192RD	I Rack	I Moody	I JHHA	I JHHB	II D95	II R36A	II R36N	II R36ND	II BW
I-192R (Type 1M)	++++	++++	+	++++	-	-	+++	-	-	-	-	-
I-JHHAR (Type 2M)	-	-	-	-	-	++	-	++++	++++	++++	++++	+++
II-R36T (Type 2'M)	-	-	-	-	-	-	-	-	-	-	-	-
II-BWR (Type 2M)	-	-	-	-	-	-	-	-	-	-	-	-
III-A66R2 (Type 3M)	-	-	-	-	-	-	-	-	-	-	-	-
VIII-R13 (Type 8M)	-	-	-	-	-	-	-	-	-	-	-	-

Species Specificity of Pneumococcus C Carbohydrate.—In all the pneumococcal strains tested, regardless of colonial morphology, the presence of the species-specific C carbohydrate could be demonstrated. For this purpose, the use of the Lancefield extract fraction precipitated by concentrations of alcohol between 70 per cent and 83 per cent and redissolved in normal saline proved most suitable (Table III). Although solutions prepared in this manner reacted occasionally with C-absorbed anti-C rabbit sera, the reactions were always less intense than with unabsorbed sera. No cross-reactions of pneumococcus C carbohydrate were noted with sera containing antibodies against the C carbohydrates of β -hemolytic streptococci of Lancefield's groups A, B, C, D, E, F, G, H, and L; nor were reactions noted between pneumococcus C carbohydrate antisera and extracts of *Streptococcus MG*, *Streptococcus salivarius*, and a strain of *Streptococcus mitis*. Antisera prepared in either the horse or the rabbit can be used to test for the presence of C carbohydrate and the test appears to be a useful aid in the recognition of unencapsulated pneumococci.

Chemical Properties of Pneumococcus M Protein.—That the type-specific substance present in unencapsulated as well as in encapsulated pneumococci is a protein similar in its properties to those of the M protein of group A streptococci

(14) is revealed by the following data. The M protein can be extracted from pneumococcal cells by hot acid solutions in which it remains soluble. It is soluble in acid ethyl alcohol but is precipitated by ethyl alcohol from neutral solutions. The serological reactivity of the material is destroyed by proteolytic enzymes as revealed in the following experiment:—

To 12.5 cc. of an extract of pneumococcus I-SV1 was added 12.5 cc. of 95 per cent ethyl alcohol. The precipitate was collected by centrifugation, redissolved in 6 cc. of acid saline, and the pH adjusted to 1.95. To two 2 cc. aliquots of the solution were added respectively 2 cc. of active and of heat-inactivated 2 per cent solutions of commercial crude pepsin, pH 2.5. After incubation for 2 hours at 37°C., the solutions were neutralized with 1 : NaOH and

Extracts and Anti-M Sera

III Acg	III SV3	III RacK	III L	III H	IV	V	VII	VIII H	VIII Marino	VIII Romano	VIII RHH	IX	XA	XIV	XVII	XIX	XXIVA
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
+++	+++	+++	+++	+++	-	-	+	++	+++	+++	+++	-	-	-	-	-	-

cleared by centrifugation. Serological reactions of the materials treated in these ways are shown in Table IV.

The serological activity of the type-specific substance is lost almost completely after 2 hours' exposure to an active solution of pepsin. Similar results are obtained with the use of commercial crude trypsin.

Like the M proteins of group A streptococci, the type-specific proteins of pneumococci form acetone-soluble picrates. When the procedure of picrate precipitation outlined in a preceding section is carried out, partially purified preparations of M protein of high serological activity can be obtained from extracts of encapsulated or of Griffith rough variants. One such preparation, when examined with the ultraviolet spectrophotometer, contained approximately 98 per cent protein, 2 per cent nucleic acid. Whether or not pneumococcal M protein is antigenic when treated by these methods has not yet been determined.

TABLE III

Precipitin Reactions of Ethanol-Precipitated Fractions of Lancefield Extracts of Pneumococci with Pneumococcus Anti-C Carbohydrate Rabbit Antiserum before and after Absorption with C Carbohydrate

Serum	Extract dilution (final)	Extract															Controls	
		I SV1	I 192R	I 192RD	II D39S	II R36A	IV	V	VII	IX	XA	XII	XVII	XIX	XX	XXIVA	C carbohydrate*	Saline
Pneumococcus anti-C rabbit serum	1:2	++	++	+	++	++++	++	+++	+++	+++	+++	+++	+++	+++	++	++	++++	-
	1:20	+	+		±	++	±	±	+	++	+	+	++	++	±	+	+++	-
C-absorbed pneumococcus anti-C rabbit serum	1:2	+	-	-	-	++	-	-	-	+	-	-	-	-	-	-	-	-
	1:20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* Pneumococcus C carbohydrate solution 1:25,000 diluted 1:2 and 1:20. Extract fraction precipitated by concentrations of alcohol between 70 and 83 per cent. Precipitin reactions performed with test tube technique.

TABLE IV

Effect of Pepsin on M Protein of Pneumococcus M Precipitin Reactions

Extract I-SVI	Type 1 M rabbit antiserum
Untreated extract	++++
Pepsin-treated extract	±
Inactive pepsin-treated extract	+++±
Saline control	-

Precipitin reactions performed with test tube technique.

TABLE V

Agglutination of Griffith Rough Pneumococci by Pneumococcus Type 1 M Rabbit Antiserum

Vaccine used in agglutination test	Antiserum* dilution									Saline control
	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10,240	
I-192R	++	++++	++++	++++	++++	++++	++++	++++	-	-
II-R36A	++	+	-	-	-	-	-	-	-	-
III-A66R2	++	+	+	-	-	-	-	-	-	-

* Antiserum prepared against strain I-192R and absorbed with strains II-R36A and III-A66R2.

homologous M protein purified partially by the picrate method so that it gives no longer a positive precipitin reaction, it agglutinates the homologous strain less well than previously but to a higher titer still than the heterologous strains.

Whether this finding indicates that the treatment of the M protein has altered it appreciably from its native state, the incomplete absorption of M antibody or the presence of an additional type-specific agglutinin cannot be stated at this time. M protein antisera do not agglutinate encapsulated variants of the homologous strain nor do they give rise to a quelling reaction with homologous encapsulated or unencapsulated organisms.

TABLE VI

Loss of Agglutinability of Pneumococcus I-192R by Homologous Anti-M Serum Following Exposure to Pepsin

Vaccine used in agglutination reaction	Antiserum* dilution								Saline control
	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10,240
Pepsin-treated cells 2 hrs. at 37°C.	+	-	-	-	-	-	-	-	-
Inactive pepsin-treated cells 2 hrs. at 37°C.	++++	++++	++++	++++	++++	++++	++++	+	-

* See footnote to Table V.

TABLE VII

*Disappearance of M Protein from Pneumococcal Cells Following Exposure to Pepsin
M Precipitin Reactions with Cells of Pneumococcus I-192R*

Extract from:	Antiserum vs. pneumococcus I-192R
Pepsin-treated cells 2 hrs. at 37°C.	-
Inactive pepsin-treated cells 2 hrs. at 37°C.	++++

Capillary tube technique.

Effect of Proteolytic Enzymes on the M Protein of Intact Pneumococcal Cells.—As is true of the M proteins of group A streptococci (15), those of pneumococcus are susceptible in the intact cell to the action of proteolytic enzymes. When heat-killed pneumococcal cells are subjected to the action of commercial crude pepsin, their agglutinability by homologous anti-M serum is almost completely lost and no M protein can be demonstrated in extracts made from cells so treated (Tables VI and VII). The morphology of the cells and their reaction to the Gram stain are unaltered by exposure to the enzyme under these conditions.

Loss of Protective Effect of Pneumococcal M Protein Antisera.—M protein antisera appear not to have an important effect upon the virulence of homologous encapsulated pneumococci. Mice inoculated intraperitoneally with 1 cc. of antitype 1 M serum 24 hrs. before intraperitoneal injection with a

10^{-7} dilution of a fully virulent culture of pneumococcus I-SV1 failed to survive longer than mice injected with normal rabbit serum or than untreated controls (Table VIII).

Absence of Cross-Reactions among Pneumococcal and Streptococcal M Proteins.—Because of the similar chemical properties of the type-specific M proteins of pneumococci and of group A streptococci, extracts of pneumococcus strains I-192R, II-R36A, III-A66R2, and VIII-R13 were tested with antisera against group A streptococci types 1, 3, 5, 6, 14, 17, 18, 19, 23, 24, 26, 28, 30, 31, 36, and 42 and absorbed sera against the same pneumococcus strains were tested with extracts of group A β -hemolytic streptococci types 1, 2, 3, 4, 5, 11, 12, 13,

TABLE VIII

Failure of Anti-M Serum to Protect Mice Infected with Homologous Encapsulated Pneumococci

Strain of pneumococcus	Amount of culture injected	Serum injected	D/S*
	cc.		
I-SV1	10^{-7}	1 cc. type 1 M rabbit antiserum	12/0
I-SV1	10^{-7}	1 cc. normal rabbit serum	12/0
I-SV1	10^{-7}	None	12/0
I-SV1	10^{-8}	None	5/1

Serum injected intraperitoneally 24 hours before inoculation intraperitoneally with pneumococcus culture. Type 1 M antiserum prepared against strain I-192R, absorbed with C carbohydrate.

* D/S, ratio of dead to surviving mice.

14, 15, 17, 19, 22, 23, 24, 26, 28, 29, 30, 31, 33, 36, 37, 41, 43, 44, and 46. No cross-reactions were noted.

DISCUSSION

The present studies reveal the hitherto unrecognized type specificity of a protein antigen of pneumococcus. This antigen is present in both encapsulated and unencapsulated colonial variants and unlike the analogous type-specific protein of several groups of streptococci (12), it is found in most cultures of Dawson rough variants. The M proteins of pneumococcus are an important factor in determining the antigenic heterogeneity of unencapsulated members of this species. All their chemical properties that have been studied so far appear similar to those of the M proteins of group A streptococci. The proteins of both species are soluble in hot 0.2 N acid, and in acid ethyl alcohol and the members of both groups form acetone-soluble picrates. The M proteins of both species appear to be located at or near the surface of the cell because they are destroyed by the action of proteolytic enzymes which do not produce concomitant morphological changes, and in both species they function as agglutinogens and precipitinogens. Despite these many similarities, no cross-reac-

tions have been noted yet between pneumococcal and streptococcal M proteins but it does not seem unlikely that such may be found.

Unlike the M proteins of group A streptococci, the M proteins of pneumococcus appear to play a negligible rôle in the determination of pneumococcal virulence. Evidence of this fact may be drawn from the inefficacy of anti-M serum in protecting the white mouse against infection with encapsulated pneumococci of the homologous type. Further evidence of this negative correlation will be reported in the following paper (10).

Examination of a number of strains of pneumococci of the same capsular type shows that the same capsular carbohydrate may be associated in different strains with dissimilar M proteins. This observation suggests that the synthesis and functions of the capsular carbohydrate and M protein may be unrelated. It creates also a taxonomic problem. Because the same or closely similar proteins seem to be present in organisms of the same capsular type isolated recently, it is suggested tentatively that the protein occurring in such organisms be given the Arabic number corresponding to the Roman numeral designating the capsular type. Proteins found subsequently to be associated with the same capsular carbohydrate may be designated by prime, double prime, or triple prime notations in the order of their identification. The association of different M proteins with pneumococci of the same capsular type is a finding of potential interest in the study of pneumococcal disease, for this phenomenon may provide a tool for the recognition of dissimilarity of organisms of the same capsular type which manifest differences in epidemiological behavior.

The presence of the M protein in pneumococcus provides an additional feature of similarity between members of this species and the streptococci. In addition, it has been shown that the C carbohydrate of pneumococcus can be used for identification of this species in a manner similar to that employed in the classification of certain of the streptococci. These observations lend support to those who would classify pneumococcus in the genus *Streptococcus* rather than under the separate generic name *Diplococcus*.

SUMMARY

The isolation and characterization of a type-specific M protein from pneumococcus are described. This protein is similar chemically in all respects studied to the M proteins of group A streptococci. No immunological cross-reactions have been observed, however, between M proteins of the two species.

Strains of capsular type I pneumococcus have been encountered which contain different M proteins. The same is true for capsular type II pneumococcus. It is apparent, therefore, that the capsular polysaccharides and M proteins vary independently of each other.

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ACQUISITION OF M PROTEIN BY PNEUMOCOCCI THROUGH TRANSFORMATION REACTIONS*

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Since the initial observations by Griffith (1) of the transformation of pneumococcal capsular types in the mouse, considerable progress has been made in elucidating the mechanism whereby this specific cellular alteration is effected. The studies of Avery, MacLeod, and McCarty (2) have shown that acquisition of the ability to produce a different capsular carbohydrate is dependent upon the application to unencapsulated pneumococcal cells of highly polymerized desoxyribonucleic acid. More recently, Boivin and his coworkers (3) have shown that an analogous transformation can be induced in *E. coli* with surface carbohydrate as the acquired character and have brought evidence that the agent responsible for the change is desoxyribonucleic acid. Weil and Binder (4) using culture filtrates, were able occasionally to produce type transformations of three strains of *Shigella paradysenteriae* but no information is available concerning the chemical nature either of the principle inducing the transformation or of the acquired character. To the present time, therefore, no clear evidence has been obtained of the transformation of bacterial cells with a protein as the acquired character.

In the present paper, transformation of pneumococci *in vitro* and *in vivo* with the acquisition of a different M protein (5) is reported. In addition, the independent variability of M protein and specific capsular polysaccharide is described.

Materials and Methods

Transformation Reactions.—The techniques employed in preparing transforming extracts and vaccines were those described by MacLeod and Kraus (6). Transforming extracts and vaccines prepared from pneumococcus strains I-SVI and III-A66 were used. Transformation reactions *in vitro* were carried out by the method of Griffith (1). For transformation reactions *in vivo*, the technique of Avery *et al.* (2) was employed with the following modification. When cultures of pneumococci with *H* variants on solid media were used as the source of inoculum, the tubes containing the transforming system were inoculated by means of a straight needle which had been touched to a peripheral portion of a colony. When pneumococci were grown in liquid media, the inoculum was a 10⁻⁴ dilution of an 18 hour culture.

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Strains of Griffith Rough and Dawson Rough Pneumococci.—Pneumococcus strain II-R36NC (Griffith rough) and two derivatives of it were used as inocula in most of the experiments reported. One derivative strain, II-R36N1_a, was obtained by transferring strain II-R36NC daily for 43 transfers in broth containing 1 per cent absorbed antiserum directed against the M protein of the parent strain. Lancefield extracts of this strain, II-R36N1_a, gave only faint reactions with anti-M sera that reacted strongly with extracts of the parent strain, II-R36NC. Strain II-R36N1_a retains the colonial appearance characteristic of Griffith rough pneumococci. A Dawson rough variant of strain II-R36NC, selected according to Dawson's method (7), was also used. Plates of neopeptone-meat infusion agar containing 5 per cent defibrinated rabbit blood were inoculated from a broth culture of strain II-R36NC with a fine wire in such a manner that colonies were spaced at a distance of 0.5 to 1.0 cm. The plates were incubated for 7 to 14 days at 37°C. After 5 days, rough, flat, fan-like excrescences similar to those described by Dawson were noted at the periphery of some colonies. Portions of a number of these outgrowths were inoculated by means of a straight needle into tubes of neopeptone-meat infusion broth and incubated overnight. Only those broth cultures showing growth characterized by complete autoagglutination were used to inoculate a second series of plates. In Dawson's experience, cultures of the rough variants of pneumococci obtained by this process of selection showed a strong tendency to revert spontaneously to the Griffith rough colonial form when cultivated in nutrient broth (7). By repeated selection and inoculation from those colonies showing the least capacity for reversion, however, strains of Dawson rough organisms were obtained after 10 serial transfers which showed little or no tendency to revert spontaneously to the Griffith rough form even after 5 or 6 rapid serial transfers in a highly nutrient liquid medium. In Lancefield extracts of such relatively stable Dawson rough strains, the M protein characteristic of the parent culture could still be demonstrated, but it appeared reduced in amount. In view of Dawson's observation that reversion of colonial type is favored by transfer in nutrient broth, inocula of the Dawson rough strain R36ND₁₁, used in the present transformation experiments, were obtained by touching a needle to the desired portion of the colony on solid medium. This inoculum was then transferred directly to tubes containing the transforming system.

In addition to the pneumococcal strains described above, in certain experiments another Griffith rough variant, II-R36A, derived from the encapsulated type II strain II-D39S, was used as inoculum.

In the designation of transformed strains the following set of conventions has been used: the designation of the parent strain is followed by a dash, the letter M and an Arabic numeral to indicate the type of M protein, and the letter S and a Roman numeral to indicate the type of capsular carbohydrate possessed by the cell. For example, strain II-R36NC-M2'SIII is a strain of pneumococcus derived from capsular type II possessing the type-specific protein (M2') of the parent type II cells and the capsular carbohydrate (SIII) of type III. Similarly, strain II-R36NC-M1SIII is one derived from capsular type II possessing the type-specific protein (M1) of type I and the capsular carbohydrate of type III (SIII).

Preparation of Anti-M Sera, M Extracts, and Techniques of Precipitin and Agglutination Tests.—The methods used were those described in the preceding paper (5).

EXPERIMENTAL

1. Transformation Reactions of Pneumococci with Acquisition of Capsular Polysaccharide but without Acquisition of M Protein.—Because pneumococcus strains II-R36NC and II-R36A had been shown previously to be susceptible to transformation reactions involving the acquisition of capsular polysaccharides, these two Griffith rough variants of pneumococcus II-D39S (capsular type II)

were selected for study. The results of *in vitro* transformation of these two strains to capsular type III and of strain II-R36A to an intermediate variant of capsular type I are shown in Table I. Although capsular polysaccharides have been acquired, the type-specific protein remains that of the parent strain, demonstrating the independent variability of the two cellular components. Strain II-R36A-M2'SI(int) is of further interest because of its low virulence for mice. Pneumococci of this intermediate strain, possessing a very small, but detectable capsule of type I polysaccharide, kill mice only when amounts of 16 hour culture as large as 1×10^{-1} to 1×10^{-2} cc. are injected. In view of the fact that this strain was derived from strain II-D39S which is fully virulent for mice (M.L.D. = 1×10^{-5} cc. of a 16 hour broth culture) and because both

TABLE I

Antigenic Composition of Pneumococcus Strains II-R36NC and II-R36A and of Derivative Strains Obtained by Transformation Reactions in Vitro

Strain	Source of transforming extract strain	M protein	Capsular SSS
II-R36NC	—	2'	None
II-R36NC-M2'SIII	III-A66	2'	III
II-R36A	—	2'	None
II-R36A-M2'SIII	III-A66	2'	III
II-R36A-M2'SI(int)	I-SVI	2'	I

strains possess the same M protein, the findings suggest that the M protein of pneumococcus plays a minor rôle in the determination of virulence. This observation is in accord with the one noted in the preceding paper that anti-M sera afford little or no protection against infection with homologous encapsulated organisms.

2. *Transformation in Vitro of Pneumococci with Concurrent Acquisition of M Protein and of Capsular Carbohydrate.*—In none of the experiments performed *in vitro* with strains II-R36NC or II-R36A was the acquisition of M protein demonstrated. Inasmuch as these two strains possess M2' protein, this finding may not be altogether remarkable. Although transformation of pneumococci of one capsular type in the mucoid or encapsulated phase directly to the mucoid phase of another capsular type has been reported (8), it seems probable that the transformation occurred because of the appearance during growth of unencapsulated mutants derived from the original mucoid strain. The cells of these unencapsulated variants, lacking presumably the characters requisite for the production of capsular polysaccharide of any type are susceptible to transformation upon exposure in a suitable environment to deoxyribonucleic acids from the homologous or heterologous types. When transformation takes place, the altered cells again produce capsular carbohydrate, the type being determined by the nature of the nucleic acid.

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It was reasoned by analogy, therefore, that if it were possible to select cells deficient in M protein, these cells might be capable of acquiring this cellular component through the transformation reaction. To obtain cells deficient in M protein, pneumococcus II-R36NC was grown in neopeptone broth containing 1 per cent of a potent anti-M2' protein rabbit serum which had been absorbed with strains I-192R and III-A66R2 to remove species-specific antibodies. Lancefield extracts of cells obtained from the sixteenth daily transfer in antiserum broth and of cells from the same culture transformed to capsular type III showed still the original M2' protein. Extracts of cells from the thirty-fourth transfer showed, however, an apparent reduction in the amount of M2' protein produced by the culture, though its presence was still detectable. When cells from the forty-third transfer, strain II-R36N1₄₃, were tested in transformation reactions with an extract of strain III-A66, colonies were obtained on subculture which were of capsular type III. Of two colonies tested, one gave rise to a strain possessing type 2'M protein. The cells from the other colony, however, were found on subculture to possess type 3 M protein as well as type III SSS. The parent cell of this culture had undergone apparently, a double transformation with acquisition of SSS III and M3 protein.

An alternative method for obtaining cells deficient in M protein was sought by growing rough variants of pneumococcus II-R36NC according to the method of Dawson described in the section on methods. Transformations to capsular type III of Dawson rough variants from serial transfers 3, 5, and 8 showed no concomitant acquisition of M3 protein. On the eleventh serial transfer of the Dawson rough variant derived from strain II-R36NC, examination of Lancefield extracts made from these cells suggested a diminution in the quantity of M2' protein produced by the culture. Cells from several colonies of the eleventh passage, strain II-R36ND₁₁, were tested in transformation reactions with an extract made from pneumococcus III-A66. Following incubation at 37°C. for 24 hours, samples were plated on blood agar. Examination of these platings revealed three colonial forms: mucoid, Griffith rough, and Dawson rough. Control cultures showed only the Dawson rough colonial phase initially and after 6 serial subcultures. Griffith rough and mucoid colonies from several cultures were selected and grown for the preparation of Lancefield extracts. Extracts of cultures of most of the mucoid colonies (capsular type III) showed M2' protein. One mucoid colony, however, gave rise to a culture possessing M3 protein in addition to the capsular polysaccharide of type III. It is of interest that a Griffith rough strain recovered from the same culture contained M2' protein although it had undergone alteration in colonial morphology from the Dawson rough form.

By two independent methods of selection, therefore, strains of pneumococcus were obtained which are capable of acquiring through transformation reactions

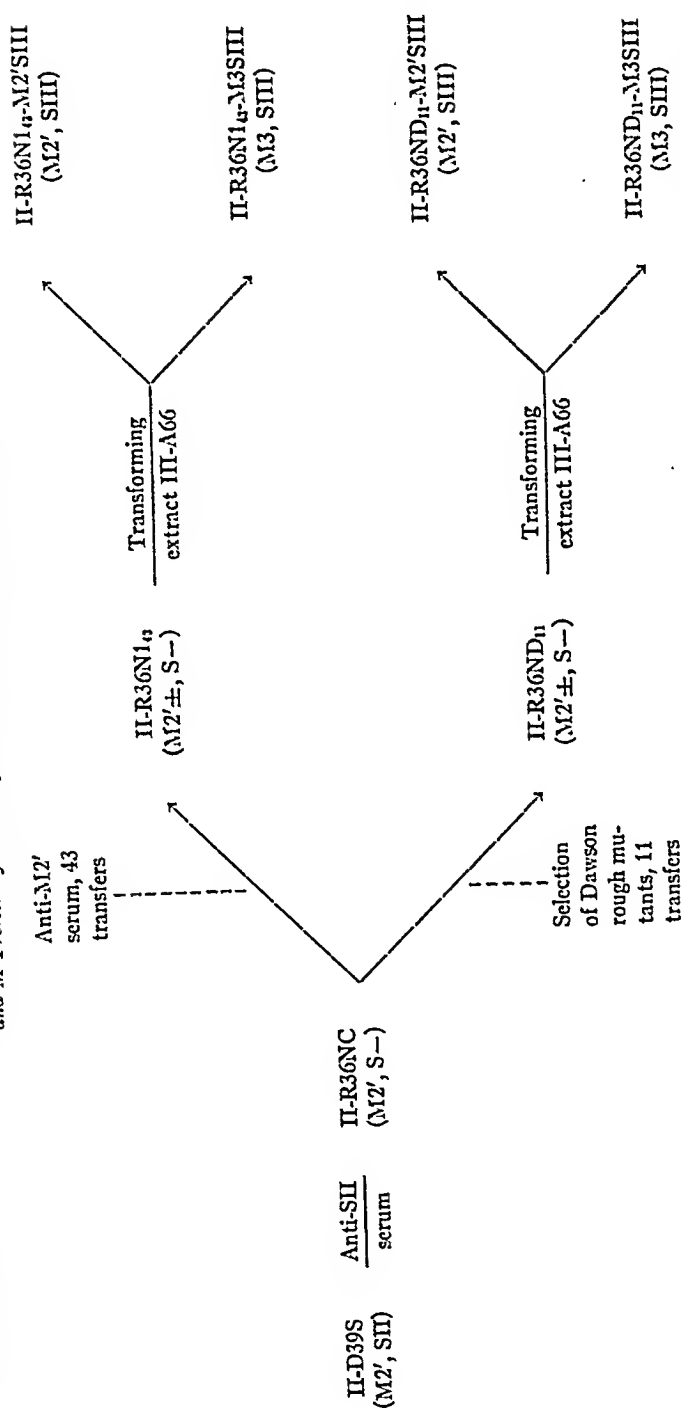
in vitro, a type specific M protein different from that possessed by the original type II strain from which they were derived. The results of these experiments are summarized schematically in Fig. 1 together with an antigenic analysis of the strains concerned.

3. Transformation of *Pneumococci in Vivo*, with Concomitant Acquisition of M Protein and of Capsular Polysaccharide.—Transformation of pneumococci with acquisition of M protein was accomplished *in vivo* by the method of Griffith. Mice were inoculated subcutaneously with a live culture of the Griffith rough strain, II-R36NC, together with a heat-killed vaccine of encapsulated pneumococci prepared by the method of MacLeod and Krauss (6). In none of the experiments were viable organisms detected in the transforming vaccines, either by mouse inoculation or by culture. Transformation of pneumococcus II-R36NC to capsular type I was induced by a vaccine prepared from pneumococcus I-SVI in eight of ten mice inoculated. Transformation to capsular type III was effected with a vaccine of pneumococcus III-A66 twice in ten attempts. In one instance of transformation to each capsular type, concomitant acquisition of M protein occurred. The strains in which the acquisition of two new characters took place differed distinctively in their colonial morphologies from those of the strains from which the transforming vaccines were made, whereas capsular transformations of pneumococcus II-R36NC possessing either the M protein of that strain or the M protein of the transforming vaccine were indistinguishable from each other in their colonial appearance. In view of these morphological observations and the absence of viable organisms from vaccines, there can be no doubt that a different M protein can be acquired through transformation carried out *in vivo* by the technique of Griffith.

4. Combination of Three Inheritable Characters within a Single *Pneumococcal Strain*.—To determine whether a strain of pneumococcus possessing the colonial properties of one type and the M protein of a second type could be transformed so that it now produced the capsular polysaccharide of a third type, the following experiments were undertaken. Pneumococcus strain II-R36NC-M1SI, which had acquired M1 protein and SSSI through transformation *in vivo* of pneumococcus II-R36NC in the presence of a transforming vaccine of pneumococcus I-SVI, was grown in 20 per cent antipneumococcus capsular type I serum broth. A Griffith rough variant containing M1 protein was obtained after eight transfers. This variant was then exposed *in vitro* to a transforming extract of pneumococcus III-A66. Platings of this culture revealed encapsulated pneumococci which on analysis showed the basic colonial morphological properties of strain II-R36NC, the type I M protein of strain I-SVI, and the type III capsular polysaccharide of strain III-A66. It is possible, therefore, by transforming reactions to combine three inheritable characters of three previously distinct pneumococci within a single cell. (Fig. 2)

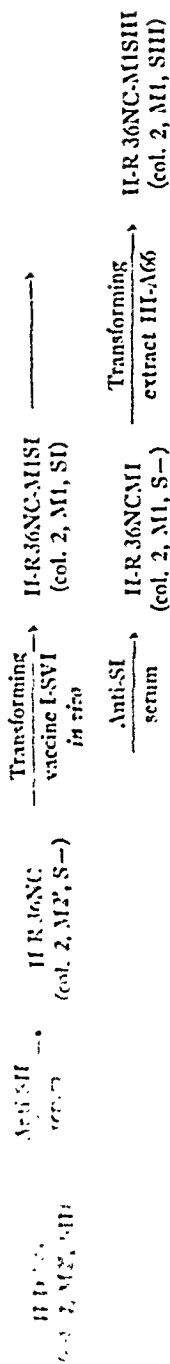
FIGURE 1

Methods of Selecting Rough Variants of Pneumococcus Strain IL-D39S and of Directing the Acquisition of New Specific Capsular Polysaccharide and M Protein by Means of Transformation Reactions in Vitro



Antigenic analysis of strains is contained in parentheses below strain designations. The type-specific protein is indicated by the letter M and the Arabic numeral following it, the capsular polysaccharide by the letter S and Roman numeral following it. Absence of the antigen is indicated by —, a culture containing probably an increased proportion of deficient cells by ±. Notations above and below the arrows indicate the method used in selecting variants and the source of transforming extracts.

FIGURE 2
Comparison of Infectious Characters of Three Pneumococcal Strains within a Single Strain



The transformations are the same as those employed in Fig. 1. The retention of basic colonial morphological properties (autolytic and hemolytic) of the parental strain in the ensuing transformations is indicated by the inclusion of the symbol col. 2 in the parentheses below the strain designation.

DISCUSSION

The studies described in this paper demonstrate that the M protein of pneumococcus can be acquired through the transformation reaction and add a new class of biological compounds, the presence and type specificity of which are subject apparently to the control of highly polymerized desoxyribonucleic acids. Transforming extracts are capable of causing the acquisition, not only of the capsular polysaccharide of pneumococcus as described previously by others, but also of the M protein and would appear capable also of inducing alteration of colonial morphology from the Dawson rough to the Griffith rough variant when the appropriate conditions exist. Inasmuch as it has been demonstrated that pneumococcus capsular carbohydrate and M protein may vary independently of one another, the results obtained suggest that transforming extracts of encapsulated pneumococci contain a multiplicity of desoxyribonucleic acids which control the specificities of the several cell characters described.

At the present time, transformation of pneumococci involving acquisition of M protein cannot be carried out with as high a frequency as can that of the capsular polysaccharides. Although acquisition of a new M protein by pneumococcus strain II-R36NC occurs occasionally in the mouse under the influence of heat-killed transforming vaccines, acquisition of a new M protein by this strain *in vitro* has not been observed. It is possible, however, that cultures of strain II-R36NC contain cells which are deficient in M protein but in such small numbers that detection of transformation is unlikely unless strongly selective factors operate. In a sense, acquisition of a new M protein by pneumococcus strain II-R36NC *in vivo* is analogous to the transformation *in vitro* of pneumococci of one capsular type directly to another capsular type reported by Dawson and Warbasse (8). It was their opinion that although unencapsulated forms were not seen in the cultures, transformation took place probably by way of these variants. If, however, pneumococcus II-R36NC is grown in the presence of anti-M2' serum or if Dawson rough variants are selected by suitable cultural methods, strains are obtained by either technique which, when extracted by hot acid solutions, yield less M protein than the parent strain. It seems probable that the methods of selection employed have increased the relative number of M protein-deficient cells in the culture and have improved the probability of detection of cells with a newly acquired M protein following exposure of the culture to transforming extracts. This hypothesis would account for the presence of capsular transformations associated with either the M protein of the cells used as transforming agents or that of the culture subjected to transformation. That only a fraction of the cells exposed to transforming extract acquire a new M protein is not remarkable if it be recalled that in all probability but a small percentage of M protein-containing, unencapsulated cells similarly treated undergo capsular type transformation.

The experiments reported here suggest that acquisition of a class of proteins by pneumococci is probably controlled by desoxyribonucleic acids. Of the transformations reported previously, all analyzed chemically have concerned the acquisition of polysaccharides. Although transformation involving the surface carbohydrate of *E. coli* (8) and of the type-specific antigens of *Sh. paradysenteriae* (4) have been accompanied by altered fermentative activities, the alterations suggest the loss of enzymes rather than their acquisition.

The demonstration of the independent variability of capsular polysaccharide and of M protein in transformed pneumococci is of interest in view of the observation in the preceding paper (5) that the same capsular polysaccharide may be associated with different M proteins in nature, and that the same or very similar proteins may occur with different capsular polysaccharides. An additional finding of interest is the fact that by the use of transformation reactions it is possible to combine within a single pneumococcal cell characters derived from three distinct pneumococcal strains. If it be assumed that the number of type-specific M proteins and of distinctive colonial forms approximates the number of known capsular types, then the possible number of antigenically distinct pneumococci is in excess of 500,000. The demonstration that it is possible to effect such combinations of antigens in the laboratory and the observations made upon naturally occurring strains suggest the desirability, whenever possible, of isolating chemically the reactive substances which enter into an antigenic analysis of bacteria in order to avoid or to elucidate "cross-reactions" which may prove confusing. That such a procedure is useful is borne out by the studies of the relations of the M and T antigens of group A β -hemolytic streptococci (9).

SUMMARY

Acquisition by pneumococcal variants of M protein and of capsular polysaccharide different from those present in the parent strain has been effected *in vitro* by means of transforming reactions with extracts of heterologous encapsulated pneumococci. Similar transformations have been accomplished *in vivo* with heat-killed vaccines as the transforming agents.

Independent variation of pneumococcal capsular polysaccharide and M protein observed in nature can be brought about also in the laboratory. By means of transforming reactions, it has been demonstrated that inheritable characters of three distinct pneumococcal strains can be combined within a single strain.

It is suggested that acquisition of M protein through transformation reactions occurs in cells deficient in that character.

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STUDIES ON THE MECHANISM OF THE SHWARTZMAN PHENOMENON*

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PLATE 21

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The intradermal injection of filtered bacterial toxin derived from certain Gram-negative microorganisms produces, in rabbits, little evidence of primary damage to the skin other than varying degrees of local inflammatory reaction. The skin may show mild erythema, usually with slight thickening of the injected area. After an interval of 12 to 18 hours, an intravenous injection of the same material causes the appearance of extensive hemorrhagic necrosis in the prepared skin site. This reaction, first described in 1928 (1), has become generally known as the Shwartzman phenomenon. No satisfactory explanation of its pathogenesis has yet been made.

Very little is known about the basic mechanisms which are implicated in cellular injury of any sort. In recent years, interest in the general problem of tissue damage has been stimulated by wartime investigations into the traumatic effects of arsenical poisons (2) and nitrogen mustards (3), which have demonstrated that certain types of tissue damage may be correlated with biochemical alterations involving cellular enzyme systems. It is probable that the various types of tissue injury caused by bacteria and their products, or by antigen-antibody reactions, will eventually become explainable at a biochemical level. As an approach to this problem, the Shwartzman phenomenon offers an experimental model in which one variety of injury can be studied from this point of view.

The phenomenon is, in a sense, non-specific. Preparation of the skin with culture filtrate from one bacterial species renders the skin susceptible to hemorrhage when filtrate from an entirely unrelated species is injected intravenously. Preparation of the skin can be accomplished by inducing local infection with certain Gram-negative bacteria, streptococci, staphylococci, or pneumococci, as well as by local skin infection (vaccinia) (4).

The capacity of antivenom-antibody combinations to bring about the phenomenon has been established in detail by Shwartzman (4). He showed that rabbit

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which had previously received horse serum exhibited typical hemorrhagic necrosis in skin sites prepared with bacterial filtrate, within 1 hour after the intravenous injection of horse serum. Other protein antigens produced similar and specific effects in appropriately sensitized rabbits. Moreover, the reaction could be induced in normal rabbits when mixtures of antigen and antibody, or the washed precipitates from such mixtures, were injected intravenously. The numerous investigations dealing with this and other features of the phenomenon have been amply reviewed by Schwartzman (4).

It is possible that in certain malignant neoplasms of animals a state of affairs may already exist which is similar to that created in rabbit skin by the local injection of bacterial toxin. It has been repeatedly demonstrated that hemorrhagic necrosis can be produced in such tumors by intravenous injection of those bacterial toxins capable of eliciting the Schwartzman phenomenon (4, 5). The mechanism by which hemorrhage is brought about in tumors has never been explained.

The hemorrhagic skin lesion which characterizes the Schwartzman phenomenon appears to be due to damage to the walls of small blood vessels. The events which precede the rupture of these vessels may be divided into two separate stages. The first stage is initiated by the intradermal injection of the bacterial toxin, and requires a period of 18 hours or so, during which time the skin becomes "prepared." There is no evidence of damage to blood vessels during this period, and the only indications of a disturbance in the tissue are edema and an infiltration by polymorphonuclear leucocytes. The second stage is initiated by the intravenous or "provocative" injection of bacterial filtrate, and consists of an injury to the blood vessels in the prepared area. One or 2 hours are required for the effects of this injury to become apparent. At this time, petechiae begin to appear in the skin, and during the ensuing 30 minutes a gross hemorrhage involving the entire prepared area occurs.

In attempting to investigate the mechanism of the Schwartzman phenomenon, it has been assumed as a working hypothesis that the preparatory injection brings about an alteration in local environmental conditions which does not of itself cause damage to the blood vessels. Under these new conditions, however, the vessels are rendered vulnerable to a second change in circumstances, which is brought about by the intravenous injection. On this basis, it appeared reasonable to approach the problem of the Schwartzman phenomenon along two separate lines, involving the mechanism of preparation and that of provocation. The first section of the present report is concerned with the results of a study of certain metabolic functions in rabbit skin following an intradermal injection of meningococcal toxin. In the second section, the effect of the intravenous injection of toxin is considered, and an attempt is made to formulate a theoretical explanation for the Schwartzman phenomenon.

Materials and Methods

Bacterial Toxins.—Most of the experiments described were performed with a meningococcal toxin which was supplied by Dr. Gregory Schwartzman. In some experiments a purified polysaccharide toxin derived from *Serratia marcescens* was employed; this material was supplied by Dr. Murray Shear. The methods used for the preparation of these materials are described in publications of these authors (4, 5).

Rabbits.—Male rabbits of hybrid white and grey stock, weighing approximately 2 kilos, were used in all experiments.

Intradermal Injection of Bacterial Toxin.—The hair over the entire abdomen was removed by shaving at least 24 hours in advance of all experiments. Meningococcal toxin was injected intradermally in a 1 to 4 dilution in physiological saline. A dose of 0.5 cc. was used in all experiments. *S. marcescens* toxin was given in an amount of 0.05 mg. contained in 0.5 cc. of saline. In the sections which follow, the term "prepared" will be used to designate skin into which bacterial toxin has been injected previously.

Intravenous Injection of Bacterial Toxin.—Meningococcal toxin was diluted 1 to 50 in physiological saline; and 2 cc. of this solution was injected intravenously into each rabbit. The dose of *S. marcescens* toxin was 0.05 mg. contained in 2 cc. of saline.

Manometric Measurements.—Eighteen hours after the intradermal injection of toxin, the animals were killed by a blow on the head. The abdominal skin was scrubbed with distilled water, dried with a cotton sponge, and strips of skin were quickly removed from the prepared site and from a normal skin area on the opposite side of the abdomen. Care was taken to include as little subcutaneous tissue as possible in the samples. The skin was then cut into small squares, each weighing approximately 50 mg., and several of these squares were accurately weighed and placed in Krebs-Ringer solution in Warburg flasks. Manometric determinations were carried out in duplicate, using the standard Barcroft-Warburg constant volume respirometer. The results are expressed in terms of microliters of gas per gram wet weight per hour. The dry weights of numerous samples of skin were measured and found to be from 19 to 24 per cent of the wet weight for the prepared skin samples, as compared with 25 to 30 per cent for normal skin. Lactic acid determinations were carried out according to the method of Barker and Summerson (6), and the results expressed as milligrams of lactic acid per gram dry weight of tissue.

Proteolytic Enzymes.—Crystalline trypsin was obtained from Armour Laboratories and employed as a 1 per cent solution in physiological saline. Partially purified, sterile papain was prepared from the commercial crude product by three successive precipitations with alcohol. The final precipitate was dissolved in saline to give a 1 per cent solution and filtered through a Seitz filter.

Sulphydryl Compounds.—Solutions of cysteine hydrochloride and BAL (2,3-dimercaptopropanol) were prepared in physiological saline and adjusted to pH 7.0 with NaOH. These solutions were freshly prepared before each experiment.

TABLE I

Skin sample	Rabbit No.	Without glucose			With glucose		
		O ₂	CO ₂	r.q.	O ₂	CO ₂	r.q.
Prepared	1	288	204	0.71	270	248	0.90
	2	364	222	0.61	325	243	0.755
	3	402	294	0.73	345	301	0.87
	4	408	294	0.72	374	310	0.83
Normal	5	476	260	0.545	475	328	0.69
	6	286	154	0.54	295	197	0.67
	7	423	297	0.70	377	294	0.78
	8	384	219	0.57	362	254	0.71

The oxygen uptake and carbon dioxide output of rabbit skin tissue which had been prepared 18 hours previously, by an intradermal injection of meningococcal toxin, compared with normal rabbit skin. Tissue squares in Krebs-Ringer solution, without glucose and with 200 mg. per cent glucose. Oxygen in the gas phase and 0.2 cc. 10 per cent NaOH in the center well. Results are expressed as microliters of gas per gram wet weight per hour.

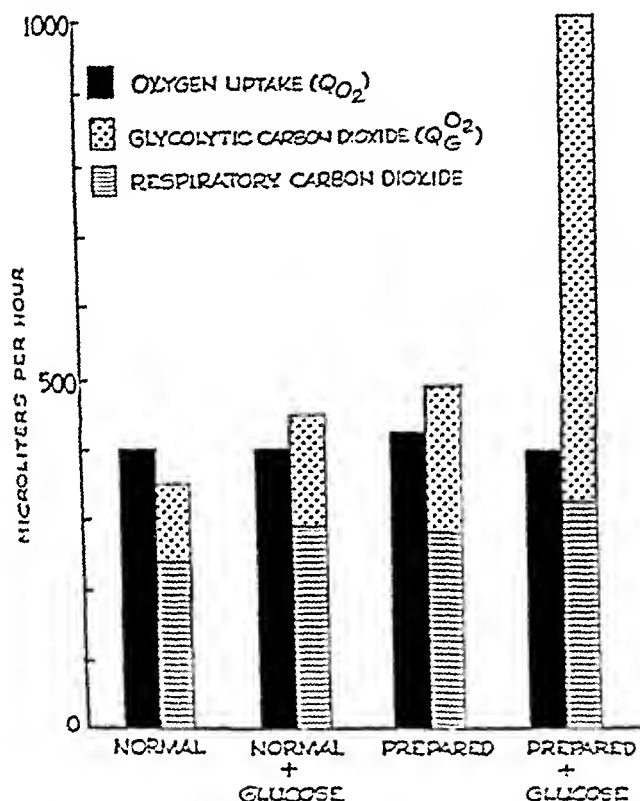
Aerobic Glycolysis of Normal and Prepared Skin.—

Samples of normal and prepared skin were placed in Krebs-Ringer solution containing added bicarbonate and glucose; and the ability of these tissues to produce lactic acid aerobically was measured by Dixon's (7) modification of Warburg's indirect method, based on the liberation of carbon dioxide from the bicarbonate buffer. Striking differences were observed between normal and prepared skin, the latter showing a twofold to fivefold increase over normal. In the absence of added glucose the differences were less pronounced but still evident. The results of a typical experiment, in which skin tissue was prepared with meningococcal toxin, are shown in Text-fig. 1, where a comparison is made between the degree of aerobic glycolysis of prepared and normal skin from a single rabbit, with and without the addition of glucose. In this figure, the tentative value for glycolytic carbon dioxide was obtained by subtraction of the respiratory carbon dioxide from the total carbon dioxide. Similar observations were made with skin tissue which had been prepared with *S. marcescens* toxin.

A summary of the determinations on prepared and normal skin samples from twenty-seven rabbits is presented in Text-fig. 2. Here the results are expressed as the ratio between the glycolytic carbon dioxide and oxygen uptake. It will be seen that this ratio was greater than unity for the majority of the prepared skin samples, and less than unity for all of the normal skin samples.

That the increased carbon dioxide output measured in these experiments was actually due to the aerobic formation of lactic acid was shown by directly determining the amount of lactic acid produced during a period of aerobic incubation in the Warburg flasks. The results are shown in Table II. It will be seen that the prepared skin samples produced four to five times as much lactic acid as did the normal tissue. In order to determine whether lactic acid

actually accumulated in prepared tissues *in vivo*, measurements of the lactic acid content were made directly on samples of prepared and normal skin which were removed from animals immediately after death and dropped at once into cold trichloroacetic acid. The results of these experiments are included in

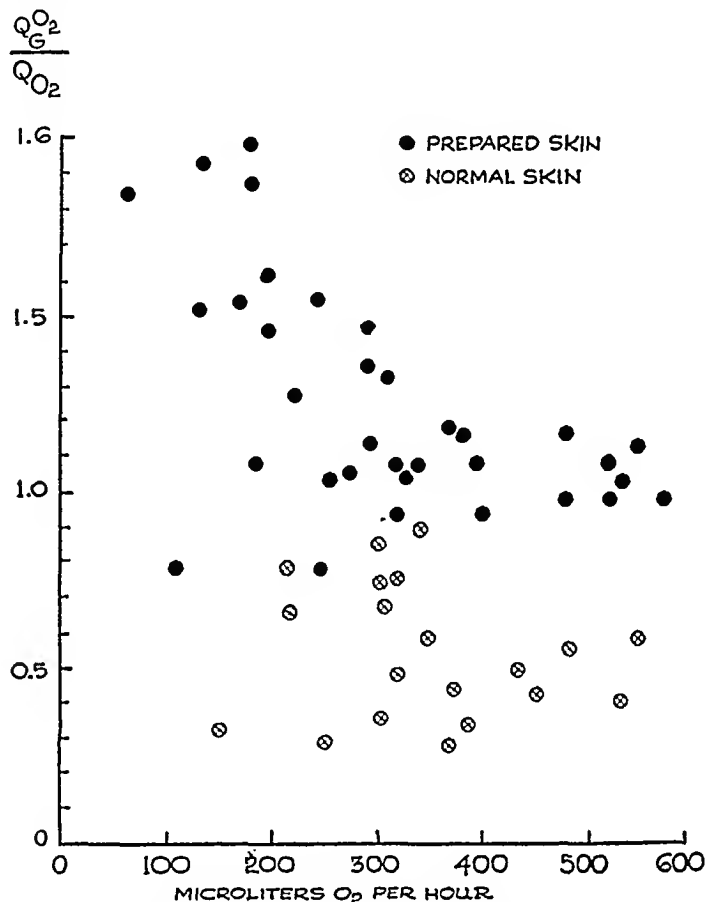


TEXT-FIG. 1. Aerobic glycolysis of normal and prepared rabbit skin, measured by Warburg's indirect method, in Krebs-Ringer solution with added 0.0014 M bicarbonate, and with 95 per cent O_2 per 5 per cent CO_2 as the gas phase. Glucose added to a final concentration of 200 mg. per cent where indicated. Results expressed as microliters per hour per gram wet weight of tissue.

Table II, in which it will be seen that the prepared skin sites contained up to five times as much lactic acid as was found in normal skin.

The increase in aerobic glycolysis was the most striking and consistent abnormality which appeared in the course of these experiments, and attempts were made to ascertain whether it was a result of some direct toxic action of the bacterial filtrate on the tissue, or a manifestation of some secondary effect. It was found that the phenomenon could not be produced *in vitro* by adding various amounts of the bacterial toxin with normal skin samples in War-

burg vessels. No increase in the degree of aerobic glycolysis could be detected, even when such mixtures were incubated for 6 hours. This is to be contrasted with another series of experiments in which bacterial toxin was allowed to act



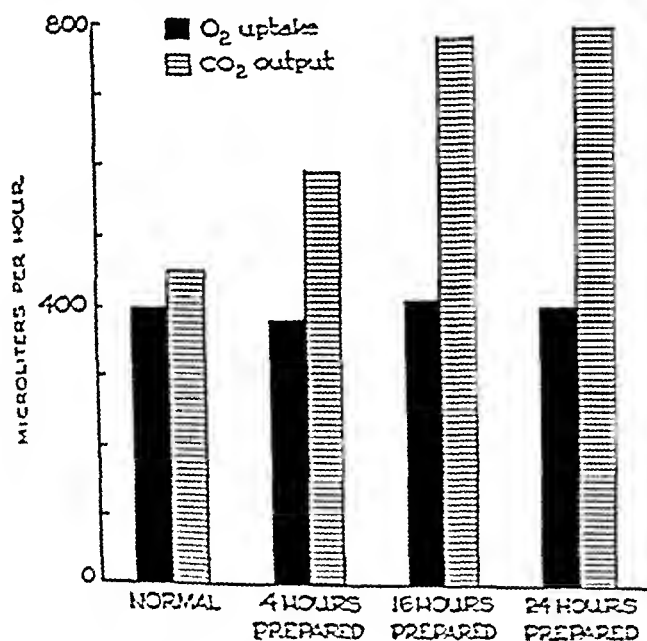
TEXT-FIG. 2. A comparison of the ratio of aerobic glycolysis to oxygen uptake in thirty-four samples of prepared rabbit skin and twenty samples of normal rabbit skin. Measurements made in Krebs-Ringer solution containing 200 mg. per cent glucose and 0.0014 M bicarbonate, in an atmosphere of 95 per cent O_2 and 5 per cent CO_2 . Oxygen uptake of each sample of tissue indicated on abscissa.

in vivo for various periods of time before the animals were sacrificed, and the skin samples removed at these times were then compared. As is shown in Text-fig. 3, some increase in aerobic glycolysis appeared as early as 4 hours after the intradermal injection, but this was less marked than in skin samples taken 16 or more hours after the injection.

TABLE II

Rabbit No.	Initial content of lactic acid		Lactic acid formed in 2 hrs. at 37°C.	
	Normal	Prepared	Normal	Prepared
1	1.7	3.56	0.02	9.74
2	0.85	4.0	3.7	23.7
3	2.0	2.75	2.8	10.75
4	1.6	8.8	4.2	14.8
5	1.4	4.5	3.1	11.3

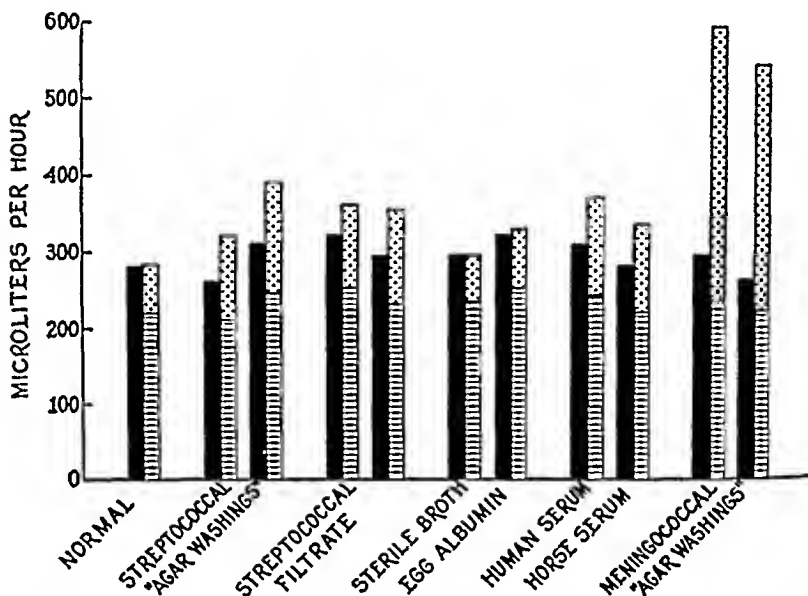
Lactic acid content of normal rabbit skin, and of prepared rabbit skin (injected intradermally with meningococcal toxin 18 hours previously), and the amount of lactic acid formed by normal and prepared skin during 2 hours at 37°C. in an atmosphere of 95 per cent O_2 and 5 per cent CO_2 . Results expressed as milligrams lactic acid per gram dry weight of tissue.



TEXT-FIG. 3. Aerobic glycolysis of normal rabbit skin compared with that of skin prepared with meningococcal toxin 4, 16, and 24 hours prior to the experiment. Krebs-Ringer solution in flask, with 200 mg. per cent glucose and 0.0014 M bicarbonate, in an atmosphere of 95 per cent O_2 and 5 per cent CO_2 . Results expressed as microliters per gram wet weight of tissue per hour.

An attempt was made to determine whether the effect on glycolysis was confined to those bacterial filtrates which are capable of preparing the skin for the Sherrisman phenomenon. Several materials which produced a visible local

inflammatory reactions in the skin but did not bring about a state of reactivity to the Shwartzman phenomenon were studied. These included fresh undiluted human and horse serum, 1 per cent egg albumin, sterile broth, filtered broth cultures of group C hemolytic streptococci, and concentrated "agar washings" of cultures of the latter organism which were prepared in the same manner as meningococcal toxin (4). The materials were injected intradermally in 0.5 cc. amounts, and 18 hours later the skin was removed and tested for aerobic glycolysis. The results of these experiments are illustrated in Text-fig. 4. It



TEXT-FIG. 4. Aerobic glycolysis of rabbit skin injected with various control substances compared with that of normal skin and of skin injected with meningococcal toxin. The experimental conditions were similar to those indicated in Text-fig. 1.

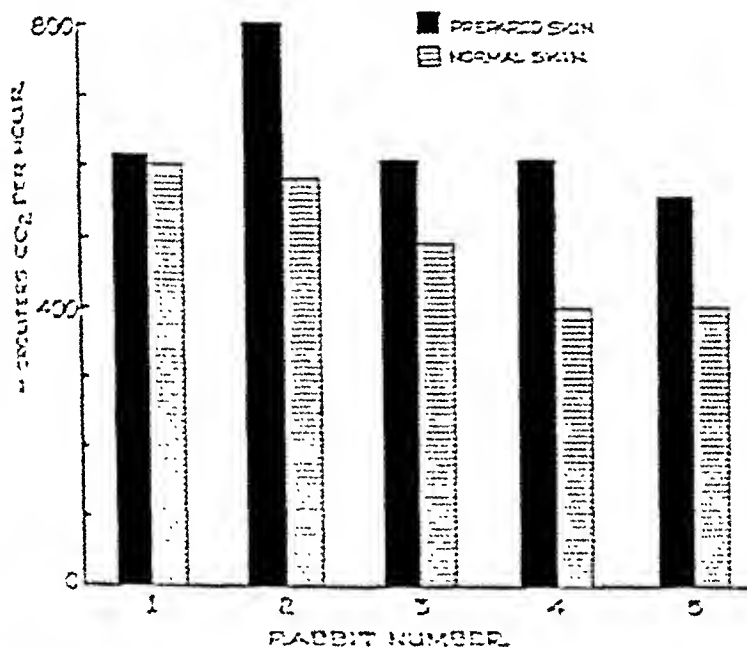
will be seen that none of these substances caused the appearance of aerobic glycolysis in significant degree. When the ratio $\frac{Q_{O_2}^{(a)}}{Q_{O_2}^{(b)}}$ was calculated for each skin sample, the results were found to lie well within the normal range shown in Text-fig. 2.

Anaerobic Glycolysis of Normal and Prepared Skin.—

Measurements of anaerobic glycolysis were carried out with samples of normal and prepared skin from twelve rabbits. The skin samples were placed in Krebs-Ringer solution with added bicarbonate and glucose, and incubated in an atmosphere of oxygen-free nitrogen and carbon dioxide. Simultaneous measurements of aerobic glycolysis were carried out with duplicate samples in each case.

It was found that the level of anaerobic glycolysis was consistently higher in prepared than in normal tissue, although the degree of difference was not as great as that encountered in aerobic glycolysis. The results of five illustrative experiments in which anaerobic glycolysis was determined in prepared and normal skin are shown in Text-fig. 5.

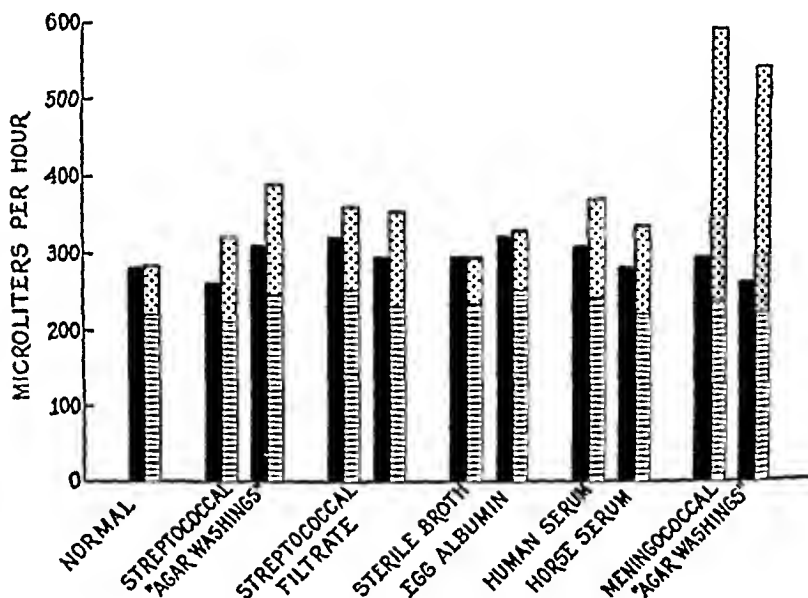
Respiration and Glycolysis Following Intravenous Injections of Bacterial Toxins.—Kun and Miller (8) have recently investigated some of the systemic



TEXT-FIG. 5. Anaerobic glycolysis of normal and prepared skin tissue in five rabbits. Measurements made in Krebs-Henseleit solution containing 200 mg. per cent glucose and 0.0014 M hydrochloric triphenyl phosphine in the gas phase. Results expressed as micromoles of carbon dioxide per hour per gram wet weight of tissue.

metabolic effects of intravenous injections of bacterial toxins similar to those used in the present study. They noted an inhibition of certain glycolytic enzymes in normal animals, which occurred within 30 minutes following the intravenous injection of pure bacterial toxin. In order to determine whether the effects were due to the toxin or to the preparation of the tissue, the authors prepared pure toxin and used it in experiments with normal animals. They observed that the toxin was highly effective in inhibiting glycolysis in normal animals, but that the inhibition was not observed in animals which had been prepared by the method of Kun and Miller (8). The authors concluded that the inhibition of glycolysis by bacterial toxin is a specific effect of the toxin on the glycolytic pathway.

inflammatory reactions in the skin but did not bring about a state of reactivity to the Shwartzman phenomenon were studied. These included fresh undiluted human and horse serum, 1 per cent egg albumin, sterile broth, filtered broth cultures of group C hemolytic streptococci, and concentrated "agar washings" of cultures of the latter organism which were prepared in the same manner as meningococcal toxin (4). The materials were injected intradermally in 0.5 cc. amounts, and 18 hours later the skin was removed and tested for aerobic glycolysis. The results of these experiments are illustrated in Text-fig. 4. It



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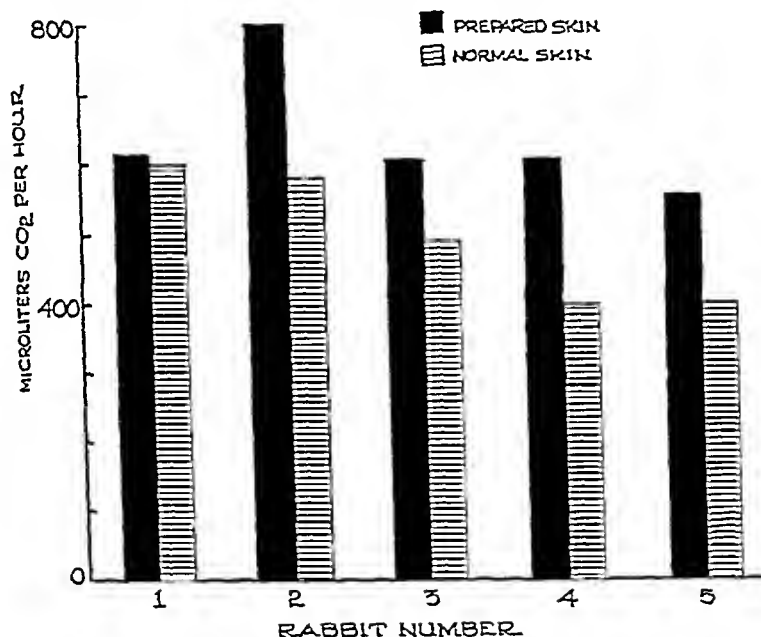
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metabolic effects of intravenous injections of bacterial toxins similar to those used in the present study. They noted an inhibition of certain glycolytic enzymes in muscle and liver, which occurred within 90 minutes following the intravenous injection of meningococcal toxin. In order to determine whether similar effects might occur in either normal or prepared skin, the respiratory exchange and glycolysis were measured in skin samples taken from rabbits at various times after an intravenous injection of meningococcal toxin, up to and including the time when hemorrhage had actually begun in the prepared skin. The respiration and glycolysis of normal and prepared skin showed no significant changes following the intravenous injection. In a few

determinations made on prepared skin which had already begun to show hemorrhage, the oxygen uptake was somewhat below normal, but this finding was not constant and may have been related to the secondary tissue damage produced by hemorrhage.

2. The Capacity of Proteolytic Enzymes to Produce Hemorrhage in Rabbit Skin, and the Effect of Intravenous Bacterial Toxin on Susceptibility to Such Hemorrhage

It has been shown in the preceding section that rabbit skin, which has been prepared for the Shwartzman phenomenon by the intradermal injection of certain bacterial toxins, exhibits a striking degree of aerobic glycolysis, as compared with normal skin. Moreover, an accumulation of abnormal amounts of lactic acid in prepared skin was demonstrated. In attempting to relate these findings to the Shwartzman phenomenon, the known influence of glycolysis upon proteolysis in tissue was taken under consideration. Rubel (9), Menkin (10), and Maver, Johnson, and Voegtlin (11) have presented evidence which indicates that in tissues which undergo glycolysis there is a concomitant enhancement of the activity of tissue proteolytic enzymes. Stoner and Green (12) have shown that proteolysis occurs in ischemic muscle as the pH of the tissue becomes decreased, and may exceed the proteolysis of autolyzing muscle. The nature of the proteolytic enzymes involved is not clear, but they are presumed to belong to the class of tissue cathepsins.

It has been suggested that the increase in proteolytic activity may be designed for the removal of foreign and necrotic material from inflamed tissues (10). In view of the potentially destructive action of tissue protease, it is conceivable that an increase in enzyme activity would also result in damage to living tissue components if the latter were not protected by an inhibitory mechanism. It was therefore of interest to determine whether the intravenous injection of a bacterial toxin, capable of provoking the Shwartzman phenomenon, affected the susceptibility of normal skin tissue to the damaging effect of proteolytic enzyme. Since it was not feasible in this laboratory to undertake direct quantitative measurements of tissue protease activity in rabbit skin, an indirect approach to this problem was adopted. The response of normal rabbit skin to the intradermal injection of known proteolytic enzymes was compared with the response in rabbits which had been given an intravenous injection of bacterial toxin 1 hour previously; *i.e.*, at the time when hemorrhage would be beginning if these animals had been prepared for the Shwartzman phenomenon. The following section is concerned with the results of these and related experiments.

The Effect of Intradermal Injection of Proteolytic Enzymes.—Twelve normal rabbits were given an intradermal injection of 0.5 cc. of 1 per cent papain, in the abdominal skin. At the same time, twelve rabbits which had received

an intravenous injection of meningococcal toxin 1 hour previously were tested similarly.

Ten of the normal rabbits showed no reaction to papain other than local edema, while two developed small areas of circumscribed hemorrhage at the injected site, measuring approximately 0.5 cm. in diameter.

Eleven of the rabbits which had previously received meningococcal toxin exhibited extensive reactions of hemorrhagic necrosis following the injection of papain. These reactions began within 30 to 60 minutes after the intradermal injection and steadily increased in size and intensity during the next 2 hours, until they involved oval or circular areas measuring 5 cm. or more in diameter. The involved skin was dark blue or black, and usually showed some denudation of the superficial layers of the skin at the center. An example of a papain reaction is shown in Fig. 1. In general, the papain lesions differed in their gross appearance from the typical Shwartzman reaction in that the hemorrhage in the former involved the subcutaneous tissues more extensively and was followed, in some instances, by complete destruction of the skin with sloughing.

Similar reactions to papain were observed in each of five rabbits which had been given *S. marcescens* toxin 1 hour previously.

The optimal period for eliciting hemorrhagic reactions with papain was between 1 and 2 hours after the intravenous injection of toxin. When papain was injected at the same time as the toxin, the reactions were smaller and occurred less frequently. When the injection of papain was delayed until more than 4 hours after the bacterial toxin had been given, the hemorrhagic reaction did not occur.

It was evident from these observations that papain was capable of imitating the effect of prior skin preparation by bacterial toxin. Papain could not be used as a skin-preparing substance in the usual sense, however; when normal rabbits were given papain intradermally and then, after 18 hours, meningococcal toxin intravenously, no hemorrhage occurred.

The effect of crystalline trypsin on the skin of normal rabbits and animals receiving meningococcal toxin was tested in similar experiments. No differences were observed in the susceptibility of the two groups of rabbits. Small areas of hemorrhagic necrosis were produced in some animals of each group by 0.5 cc. of 1 per cent trypsin. None of the animals showed extensive or progressing lesions such as were seen with papain.

The existence of papain-like enzymes, having a relatively low pH optimum and requiring sulfhydryl or other reducing agents for their activation, has been demonstrated in a large number of tissues. The increased susceptibility of skin blood vessels to papain, but not to trypsin, suggested that the intravenous injection of bacterial toxin might have the capacity of interfering with a normal inhibitory mechanism for such a tissue protease. It was therefore of interest to determine whether sulfhydryl compounds were capable of activating

such a protease *in vivo* when injected into the skin. For this purpose, solutions of cysteine and BAL were injected intradermally in normal rabbits and in animals which had been given meningococcal toxin intravenously.

It was found that 0.5 M cysteine, and 0.1 M BAL, in volumes of 0.5 cc. produced little or no reaction in the skin of normal rabbits. In contrast, each of these compounds caused the appearance of extensive areas of hemorrhagic necrosis when injected 1 hour after the intravenous administration of meningococcal toxin. Similar results were obtained in rabbits given *S. marcescens* toxin intravenously. The reactions appeared at about 30 minutes after the intradermal injection, and slowly increased during the next hour. They consisted of oval or circular areas of deep blue hemorrhage, sometimes confluent and sometimes consisting of multiple petechiae. The lesions usually measured 3 to 5 cm. in diameter. In many instances they bore a striking resemblance to the gross appearance of the Shwartzman phenomenon. Typical reactions to cysteine are shown in Fig. 2.

The cysteine and BAL reactions could only be obtained when these substances were injected between 1 and 2 hours after the intravenous injection of bacterial toxin. When they were injected at the same time, or later than 2 hours after the intravenous injection, reactions did not occur.

Smaller concentrations of cysteine and BAL produced inconstant and smaller hemorrhagic lesions. The concentrations employed in the above experiments were considerably greater than would be required for the activation of papain or other sulfhydryl-activated proteolytic enzymes *in vitro*. If the hemorrhagic reactions observed following the intradermal injection of cysteine and BAL were due to the activation of a tissue protease, one might expect these substances to produce their effect in a relatively low concentration, but the following observation suggests that high concentrations may be necessary for activation *in vivo*. Five cc. of 1 per cent papain was injected intravenously into a number of rabbits, and at the same time 0.5 cc. of varying concentrations of cysteine and BAL was injected intradermally. In every instance, extensive hemorrhagic reactions occurred within 15 minutes in areas injected with 0.5 M cysteine and 0.1 M BAL. No reactions occurred, however, at sites injected with one-tenth of these concentrations of sulfhydryl compounds.

A number of other chemical solutions were injected into the skin of normal rabbits and animals which had received bacterial toxin intravenously, in an attempt to determine whether the reaction to sulfhydryl compounds and papain represented a specific event. These substances, none of which caused hemorrhagic reactions in either group of animals, included 10 per cent glutathione, 5 per cent sodium ascorbate, 5 per cent sodium lactate, 0.5 per cent potassium cyanide, 5 per cent sodium tetrathionate, 1 per cent hydroquinone, and 1 per cent gold thiosulfate. A solution of 5 per cent calcium chloride caused severe hemorrhagic reactions, but these were of the same intensity in both groups of animals.

DISCUSSION

It has been shown that rabbit skin which is prepared for the Shwartzman phenomenon by the intradermal injection of bacterial toxin exhibits a much increased degree of aerobic glycolysis and lactic acid formation *in vitro*. Evidence that this alteration occurs *in vivo* is obtained in the finding that there is a measurable increase in the concentration of lactic acid in prepared skin. The increase in aerobic glycolysis appears as early as 4 hours after the intradermal injection of toxin, but is not maximal until approximately 24 hours have elapsed.

The presence of increased aerobic glycolysis in adult mammalian tissues has generally been considered to be a manifestation of some form of damage. It has been reported, for example, in inflammatory exudates (10), in a tuberculous lymph node (21), in local vaccinia lesions in rabbit skin (14), and in various tissues damaged by mechanical means (13). It is also a characteristic feature of certain malignant tumors (15).

The concept that the release of aerobic glycolysis may be the result of damage to or interference with the so called "Pasteur mechanism" is supported by considerable evidence (13), and it is possible that some such mechanism is involved in the phenomena described in the present report. However, the failure of bacterial toxin to produce aerobic glycolysis when mixed with normal rabbit skin *in vitro* strongly suggests that the metabolic defect seen in the prepared skin samples may be due not to a direct toxic effect, but to some secondary reaction which can occur only *in vivo*. For example, it is possible that the polymorphonuclear leucocytes which appear in prepared skin tissue are the source of the increased aerobic glycolysis, and are responsible for the observed increase in total anaerobic glycolysis. This explanation was proposed by Crabtree (14) in the case of the vaccinia lesion. Available information is not sufficient to allow any definite conclusions as to whether the number of leucocytes present in the prepared skin samples could account for the increased aerobic glycolysis exhibited by the tissue as a whole.

Control experiments have been performed in which the intradermal injections consisted of various materials which are incapable of preparing the skin for the Shwartzman phenomenon. These substances included heterologous sera, broth, and culture filtrates of a group C hemolytic streptococcus. Each of these materials produced a visible local inflammatory reaction, but in no case did such tissue show the marked aerobic glycolysis characteristic of the samples prepared with meningococcal or *S. marcescens* toxin.

The studies of other workers (9-12) indicate that increased aerobic glycolysis with the accumulation of lactic acid may provide a stimulus for the action of tissue proteolytic enzymes, or cathepsins. The possibility that cathepsins may play a rôle in certain types of tissue damage has received little consideration. The function of these enzymes in tissues under normal conditions is not known, although it is generally believed that they are concerned with protein

synthesis. Their potentially destructive effect is illustrated by the familiar phenomenon of autolysis.

In the rabbit skin area prepared for the Shwartzman phenomenon, there is no evidence of physical damage to the components of this tissue until approximately 1 hour after the intravenous injection of bacterial toxin. At this time, necrobiosis involving the leucocytes and other cellular components of the tissue is observed (4), and rupture of the blood vessels occurs. If it is postulated that a proteolytic enzyme in prepared skin is responsible for this tissue damage, it is reasonable to assume that an inhibitory mechanism prevents the action of the enzyme until after the intravenous injection of toxin.

It has been observed that the intradermal injection of a 1 per cent solution of papain, which causes little or no hemorrhage in normal rabbits, results in extensive hemorrhagic and necrotic lesions in animals which have received an intravenous injection of meningococcal toxin 1 hour previously. These lesions bear a superficial resemblance to the Shwartzman phenomenon during the early period of their development, since they are characterized by profuse hemorrhage into the skin; later, when the papain lesions become wholly necrotic and sloughing occurs, the resemblance is less evident. No increase in susceptibility of skin to the hemorrhagic effect of trypsin was observed when rabbits were given intravenous toxin.

It has also been observed that the intradermal injection of neutralized solutions of cysteine and BAL causes a severe hemorrhagic reaction, 1 hour after an intravenous injection of bacterial toxin. The reactions elicited with these compounds were primarily hemorrhagic and bore a close resemblance to the Shwartzman reaction in their gross appearance.

In the absence of information regarding the actual status of protease inhibitor in skin tissue, the interpretation of these observations is a conjectural matter. The increased vulnerability of the skin to the action of papain may be due to the impairment of some inhibitory mechanism for this enzyme, but the nature of the inhibitor and the process involved in its impairment are not known. It is possible that the lesions produced by cysteine and BAL may be due to the direct chemical trauma of these reducing agents in tissue, with subsequent activation of tissue proteolytic enzyme and destruction of blood vessels. It is of interest that similar concentrations of cysteine and BAL produced hemorrhagic necrosis when injected into the skin of rabbits which were simultaneously given intravenous injections of papain solution.

In considering possible mechanisms by which susceptibility to intradermally injected protease may be affected by intravenously injected material, certain observations made by Jobling and Petersen (16-18) may be of significance. These workers reported that the inhibitor for crude trypsin could be removed from plasma by suspensions of bacteria, starch, agar, antigen-antibody mixtures, and kaolin, either when these substances were mixed with plasma *in*

vitro or injected intravenously into living animals. The authors considered that the inhibitor was probably adsorbed by the materials employed. It is known that each of these substances is capable of producing the Shwartzman phenomenon when injected intravenously into rabbits (4), including kaolin, which has been found in this laboratory to be highly effective in doses of 1 cc. of a 10 per cent suspension (19). It is not known whether the plasma inhibitor of trypsin is implicated in the control of tissue protease activity, but the possibility that a related mechanism may be involved in the Shwartzman phenomenon merits further investigation.

The mechanism by which intravenously injected bacterial toxin may affect the susceptibility of skin to proteolytic enzymes is unknown. It is possible that the systemic effects of toxin on carbohydrate metabolism may in some fashion be related to its action in the Shwartzman phenomenon. Kun and Miller (8) have reported that the injection of meningococcal toxin in rabbits causes a profound fall in the level of glycogen in liver and muscle tissue, during the hour following injection. They were unable to account for the disappearance of glycogen either on the basis of the hyperglycemia or the increased lactic acid formation exhibited by these animals. Bier and do Amaral (20) have recently shown that the intravenous injection of suspensions of glycogen will provoke the Shwartzman phenomenon, which they ascribed to the chemical similarity between glycogen and starch. We have confirmed this observation, using rabbit liver glycogen¹ in amounts of 100 mg. contained in 2 cc. of saline (19). It is conceivable that the disappearance of tissue glycogen described by Kun and Miller following the intravenous injection of meningococcal toxin and the capacity of glycogen to provoke the Shwartzman phenomenon may be related phenomena. Studies on this aspect of the problem are in progress.

In summary, the following theoretical explanation of the Shwartzman phenomenon is tentatively proposed. The effect of skin preparation with bacterial toxin is to bring about a change in the local metabolic activity of this tissue, with increased lactic acid formation. This new environment is favorable to the action of tissue protease, which is prevented from acting upon tissue components by a protease-inhibitory mechanism. The intravenous injection of bacterial toxin causes a decrease in the effectiveness of this inhibitory mechanism. In the prepared area, tissue protease is thus enabled to act on susceptible tissue components, the blood vessels undergo damage, and hemorrhage is the result.

¹ The glycogen used in these experiments was prepared from fresh rabbit livers by prolonged extraction with boiling water followed by precipitation with ethanol. Subsequent reprecipitation from aqueous solution with ethanol did not affect its hemorrhage-inducing activity. The conditions of preparation preclude the possibility of contamination of the material with bacterial products, which Shwartzman (4) has suggested as an explanation for the activity of suspensions of starch and agar.

The speculative nature of the above concept is apparent, and the evidence which is considered to support it is mainly indirect and circumstantial. It is presented because of its possible applicability to further investigation of the Shwartzman phenomenon, as well as its possible bearing on the problem of induced hemorrhage in tumors and the problem of tissue damage caused by antigen-antibody interaction.

SUMMARY

Rabbit skin which is prepared for the Shwartzman phenomenon by an intradermal injection of meningococcal toxin exhibits, *in vitro*, a high degree of aerobic glycolysis. This metabolic abnormality is reflected, *in vivo*, by a measurable increase in the concentration of lactic acid in the prepared skin. Some increase in anaerobic glycolysis also occurs in prepared skin; this is of less degree than the increase in aerobic glycolysis. The respiratory quotient of prepared skin tends to be somewhat higher than that of normal skin, although the oxygen uptake is not significantly altered.

Gross hemorrhagic lesions which resemble the Shwartzman phenomenon are produced by the intradermal injection of papain into rabbits which have received an intravenous injection of meningococcal toxin 1 hour previously. Such hemorrhagic reactions are not observed when papain is injected into normal rabbit skin.

Similarly, hemorrhagic lesions are produced by the intradermal injection of cysteine and BAL, following an intravenous injection of meningococcal toxin.

An hypothesis to explain the Shwartzman phenomenon, which implicates tissue protease in the damage to the blood vessels of the skin, is proposed.

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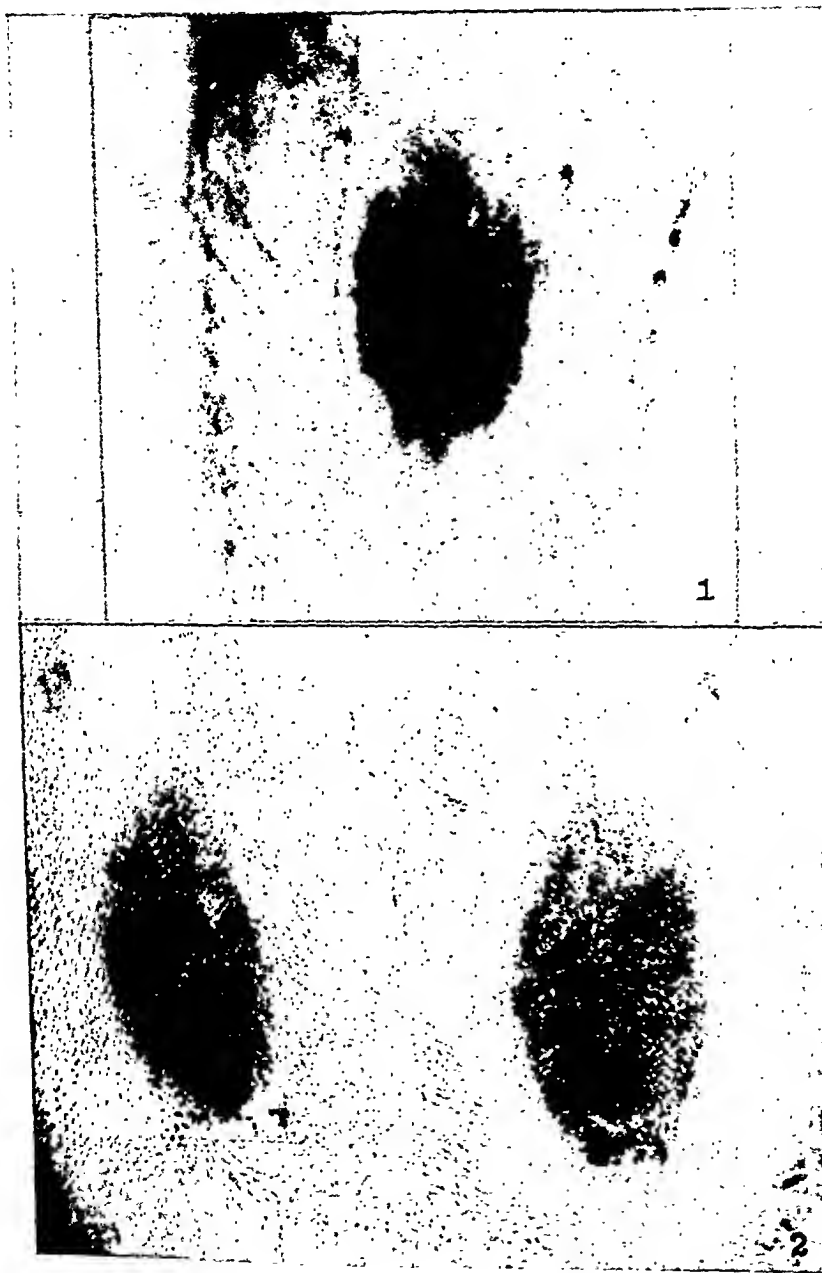
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EXPLANATION OF PLATE 21

FIG. 1. Hemorrhagic lesion produced in abdominal skin by 0.5 cc. of 1 per cent papain, in a rabbit which had received intravenous meningococcal toxin 1 hour previously.

FIG. 2. Two hemorrhagic lesions produced in abdominal skin by 0.5 cc. of 0.5 M cysteine, in a rabbit which had received intravenous meningococcal toxin 1 hour previously.



(Thomas and Stetson: Mechanism of Shwartzman phenomenon)

THE EFFECT OF LITHIUM PERIODATE ON CRYSTALLINE BOVINE SERUM ALBUMIN

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In a recent report it was shown that the biological activities of two proteins, crystalline ribonuclease and the immune globulin of Type III antipneumococcus horse serum, are destroyed when the proteins are subjected to the action of dilute lithium periodate at physiological pH values (1). The nature of the chemical changes involved are ill understood, but that they are considerable is evident not only because of the loss in biological activity which occurs, but because marked changes in the absorption spectrum are observed following the oxidation. The present paper describes a continuation of the work. In order to gain further insight into the mode of action of the periodate ion on native proteins, a study has been made of its action on crystalline bovine serum albumin. It will be shown that the physical, chemical, and immunological properties of this protein are radically altered on contact with the reagent and that these changes are accompanied by an alteration or destruction of certain amino acids.

Materials and Methods

Crystalline bovine serum albumin was purchased from The Armour Laboratories, Chicago. Buffered lithium periodate was prepared by dissolving 2.28 gm. of $\text{HIO}_4 \cdot 2\text{H}_2\text{O}$ in 50 ml. of water; 10 ml. of 0.5 N H_3PO_4 was added followed by the addition of 20 ml. of carbonate-free 1 N LiOH . The solution was then diluted to 100 ml. The pH of this solution is 7.2.

The cystine and cysteine content of the native and oxidized proteins was determined by the method of Kassel and Brand (2). The acid hydrolysis of the various proteins was carried out as described by Brand (3). Twice distilled 6 N HCl was used, and the hydrolysis was performed in the presence of urea, and in an atmosphere of CO_2 at 130–35° for 8 hours. The sample was diluted to a known volume and aliquot portions were used for the determination of the cystine and cysteine. Tyrosine analyses were performed on alkaline hydrolysates of the proteins (4) by the procedure of Lugg (5). Tryptophane analyses were carried out directly on the intact unhydrolyzed protein as recommended by Sullivan (6). Standard curves for the absorption values of the colors developed were constructed, using in each instance the pure amino acid as standard. All determinations were made in a Beckman model DU spectrophotometer. The color developed in the cystine method was read at 850 $\text{m}\mu$; in the tyrosine method 354 $\text{m}\mu$; and in the tryptophane procedure 600 $\text{m}\mu$. In all instances duplicate analyses were performed.

Electrophoretic Technique.—The electrophoretic experiments were carried out at 0.5° in the apparatus described by Longsworth (7). Samples of the native and oxidized proteins at concentrations of approximately 1.0 per cent were used. Prior to electrophoresis, the solutions were dialyzed against several portions of the same buffer as that used in the experiments.

The mobilities were computed from the descending patterns, using the bisecting ordinate of the refractive index gradient curve, and referred to 0°C.

Oxidation of Bovine Serum Albumin by Lithium Periodate.—4.0 gm. of crystalline bovine serum albumin was dissolved in 200 ml. of water; 100 ml. of 0.1 M lithium periodate buffer solution described above was then added. After standing at room temperature for 72 hours the excess periodate was decomposed by the addition of 3 ml. of 50 per cent glucose solution. The solution of the oxidized protein was thoroughly dialyzed in a rocking device against running distilled water until free of electrolytes. The end product was isolated by desiccation from the frozen state. 3.8 gm. of material was recovered. The substance was obtained as a beige-colored fluffy powder.

FINDINGS

Properties of Oxidized Bovine Serum Albumin.—When bovine serum albumin was oxidized with lithium periodate at pH 7.2, the solution gradually became yellow in color, and no visible precipitate was formed. The oxidized protein was soluble in neutral and alkaline solutions, but if the pH was lowered to pH 5.0, the solution became turbid, and at a pH of approximately 3.5 to 4.0 the protein was insoluble. The solubility of the oxidized protein in ammonium sulfate was markedly changed following oxidation with lithium periodate. At 10 per cent saturation some of the protein was precipitated, and at 30 per cent saturation the major part came out of solution. Attempts were made to crystallize the oxidized derivative from ammonium sulfate, but without success. A solution of the protein behaved in some respects like one of the native material,—the oxidized protein was precipitated by the salts of heavy metals, and by trichloroacetic acid. Solutions of the oxidized protein foamed and gave a strong biuret test. The oxidized protein was also hydrolyzed by crystalline trypsin, and to a greater degree than was the native protein when the hydrolysis was carried out under the following conditions:—

Ten ml. of a 0.1 per cent solution of native and oxidized protein was heated for 3 minutes at 70° in a 0.1 molar phosphate buffer at pH 7.6. To each solution was added 500 micrograms of crystalline trypsin, and after 18 hours at 37° an aliquot of the protein was precipitated with an equal volume of 10 per cent trichloroacetic acid. Analyses of the supernates revealed that 34 per cent of nitrogen was found in that of the heated native albumin, and 67 per cent in that of the oxidized protein. If the experiment was carried out on unheated protein solutions, the differences in soluble nitrogen were approximately of the same order, but the degree of proteolysis was one-third less.

Change in Absorption Spectrum of Bovine Serum Albumin Following Oxidation with LiIO_4 .—0.4 gm. of crystalline bovine serum albumin was dissolved in 20 ml. of water; 4 ml. was removed and saved. The remainder was treated with 8.0 ml. of 0.1 M LiIO_4 -phosphate mixture and allowed to stand at 22°. At the end of 24, 48, and 72 hours, 6 ml. samples were removed and the periodate decomposed by the addition of 0.5 ml. of 50 per cent glucose solution. All the solutions were placed in cellophane bags and dialyzed against 0.1 M phosphate buffer at pH 7.6 until free of I^- and IO_3^- . Each solution, including the control, was then diluted with the phosphate buffer until the concentration was 1.0 mg. per ml. The ultraviolet absorption spectra were determined and recorded (Fig. 1).

It is apparent from the results presented in Fig. 1 that profound changes have occurred in the ultraviolet absorption spectra of the protein after contact with the reagent. These changes become progressively greater the longer the protein is subjected to the action of the periodate ion. After 24 hour contact,

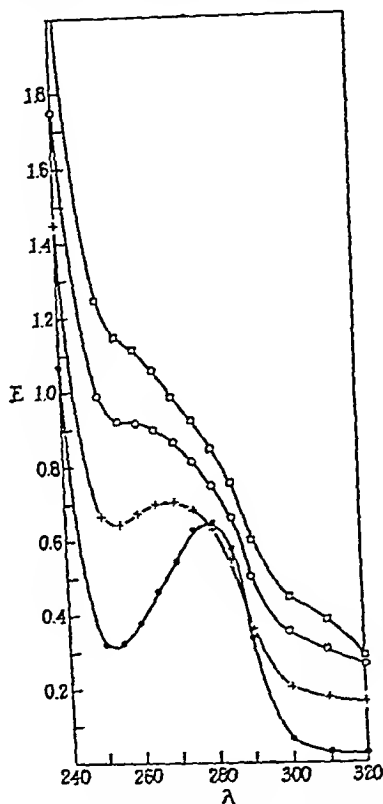


FIG. 1. Absorption spectra of bovine serum albumin before and after oxidation with 0.03 M LiIO_4 .

- = native bovine albumin.
- + = bovine albumin oxidized 24 hours with LiIO_4 .
- = " " " 48 " " "
- = " " " 72 " " "

absorption below 280 $\text{m}\mu$ is not only greater, but there is a shift of 10 $\text{m}\mu$ in the maximum. At the end of 48 and 72 hours contact with the reagent the absorption becomes progressively greater throughout the entire range of wave lengths, and there is no discernible peak. This shift in absorption spectrum is comparable to that shown by the Type III pneumococcus immune globulin after oxidation with periodate.

Determination of Amino Acids.—Because of the shift and final disappearance in the point of maximum absorption of serum albumin following oxidation with periodate, it was thought advisable to analyze the oxidized protein for its tyrosine, tryptophane, cystine, and cysteine content.

Cystine and Cysteine.—Hydrolysates (2) of the native and oxidized proteins were analyzed for their content of cystine and cysteine (3). The values are recorded in Table I. It can be seen that the cystine content of the native protein was essentially the same as that reported by other investigators (8); the cysteine content, on the other hand, was considerably lower. Repeated analyses confirmed this observation and the difference probably resides in the fact that our particular sample of bovine serum albumin had actually a lower content of labile -SH groups.

When cystine and cysteine determinations were carried out on hydrolysates of the protein previously oxidized for 72 hours, the two values were considerably lower, 2.45 and 0.13 per cent, respectively. This would indicate that a marked decrease in S-S and in -SH groupings had taken place, without a comparable loss in total sulfur, for it can be seen in Table I that the total sulfur content of the native and oxidized protein was found to be 1.90 and 1.68 per cent, respectively.

Tyrosine.—A sample of native and of oxidized bovine serum albumin dissolved in 5 N NaOH containing 20 mg. of protein per ml. was hydrolyzed in a sealed tube for 24 hours at 100° (4). After removal of tryptophane, or its products of oxidation, as the mercury salt, the tyrosine was determined according to the method of Lugg (5). The native protein was found to contain 5.63 per cent tyrosine. The hydrolysate of the oxidized protein, on the other hand, gave an intensity of color corresponding to 3.28 per cent tyrosine. From the analytical result it appears that approximately 40 per cent of the tyrosine had been destroyed or altered by treatment of the protein with lithium periodate.

Tryptophane.—Both the native and oxidized protein were next analyzed for their tryptophane content (6). The native protein was found to have a tryptophane content of 0.7 per cent, whereas the oxidized protein gave no color with the reagents, indicating that the tryptophane had been destroyed. Since color development, both in the determination of tyrosine and tryptophane, appears to be dependent upon the integrity of the phenolic and indole rings, it can be assumed that the latter have in each instance suffered chemical degradation, the extent of which is unknown.

In this connection it might be said that when pure tyrosine was subjected to the action of 0.03 M LiIO_4 at pH 7.2, approximately 1 mol of the reagent was consumed over a period of 8 hours. During the course of the oxidation the solution darkened and eventually deposited a precipitate. Chromatographic adsorption of the reaction mixture on activated alumina revealed at least four bands, indicating that a complex series of chemical reactions had taken place.

Electrophoretic Properties of Native and Oxidized Bovine Serum Albumin.—In

the foregoing account it has been shown that the oxidation of bovine serum albumin with lithium periodate causes marked changes in the chemical and physical properties of the protein. It seemed of interest, therefore, to investigate the electrophoretic behavior of the protein after treatment with this reagent.

TABLE I
*Analysis of Native and Oxidized Bovine Serum Albumin**

Analysis	Native bovine albumin	Bovine albumin after 72 hrs. oxidation with 0.033 M LiIO_4
$[\alpha]^{22}_D$	-53.0°	-69.0°
Total N, <i>per cent.</i>	16.00	14.51
Total S, " "	1.91	1.68
Cystine, " "	5.65	2.45
Cysteine, " "	0.43	0.13
Tyrosine, " "	5.63	3.28
Tryptophane, <i>per cent.</i>	0.70	0.00

* Oxidized 72 hours with 0.033 M LiIO_4 .

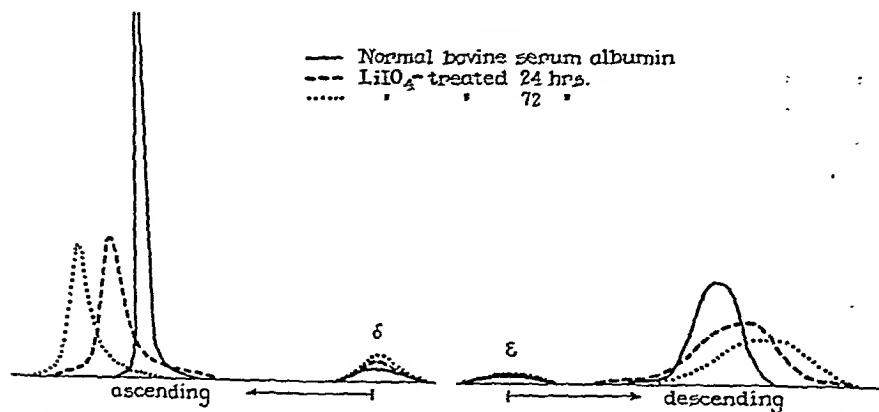


FIG. 2. Electrophoretic patterns of bovine serum albumin before and after treatment with 0.03 M lithium periodate.

In the experiment recorded in Fig. 2 the electrophoresis of bovine serum albumin before and after treatment with lithium periodate was carried out in a sodium phosphate buffer at pH 7.7, an ionic strength 0.1, at a potential gradient of 6.34 volts per cm. for 9,000 seconds. The full curve represents native bovine serum albumin, the dashed and dotted curves are patterns of the same preparation after oxidation with 0.03 M lithium periodate for 24 and 72 hours, respectively. From the figure it is apparent that a qualitative difference in the patterns exists; the boundaries of the two oxidized proteins are more

diffuse than is that of the native material. Under experimental conditions in which time and potential gradient have been kept constant, superimposition of the electrophoretic patterns permits a direct comparison of the approximate mobilities of the three protein preparations. Thus it can be seen that the mobilities of the substances are different. The actual values of $u = -6.77 \times 10^{-5}$ and $u = -7.33 \times 10^{-5}$ for the samples treated for 24 and 72 hours, respectively, are considerably higher than that of $u = -6.14 \times 10^{-5}$ of the native protein.

The large boundary spreading shown by the two oxidized proteins suggests that these preparations are less homogeneous than the native albumin. It seemed advisable, therefore, to extend the electrophoretic study over a wider range of pH values. It was observed that the protein oxidized for 72 hours migrated as a single peak at all pH values studied, except in one instance where the determination was carried out in a 0.1 N sodium acetate buffer at pH 5.64. At this pH a separation of the boundary into two peaks was observed in the ascending patterns. A comparison with bovine serum albumin in the isoelectric pH region was not possible because of the insolubility of the oxidized protein in the pH range of 3.5 to 5.2.

The results of the mobility measurements over a wide pH range of normal bovine serum albumin and of the protein treated with lithium periodate for 72 hours are presented in Table II. All the experiments were carried out in monovalent buffers of 0.1 ionic strength, the composition of which is given in column 1. The mobility values obtained for native bovine serum albumin are in good agreement with those of Longworth and Jacobsen (9). A comparison of the mobilities of the oxidized protein, column 4, with those of the native protein, column 3, shows that at all pH values below 9 the oxidized material has the more negative mobility; i.e., as a cation it migrates more slowly than the intact protein while as an anion it moves more rapidly.

In an attempt to find further differences between the two proteins, their electrophoretic behavior in a buffer containing methyl orange has also been studied. With the aid of the dialysis experiments and photometric technique of Klotz (10), it was found that the oxidized protein bound but half the quantity of methyl orange as that bound by normal bovine serum albumin. Longworth and Jacobsen (9) have shown that native bovine serum albumin has a higher mobility in a 0.1 N sodium acetate buffer at pH 5.6 containing 0.0002 M methyl orange than it does in pure acetate buffer. The difference in mobility of the oxidized protein when observed under the same conditions, $\Delta u = 0.3 \times 10^{-5}$, when compared with that of native albumin (9), $\Delta u = 0.6 \times 10^{-5}$, parallels the results obtained in the dialysis experiments.

Antigenicity of Oxidized Plasma Albumin.—In our hands crystalline bovine serum albumin has proved to be a good antigen. Rabbits injected intravenously with 5 mg. of protein for 6 consecutive days, followed by a rest period

TABLE II
Mobilities of Bovine Serum Albumin before and after Treatment with Lithium Periodate in Buffer Solutions of Ionic Strength 0.1

Buffer (1)	pH (2)	$u \times 10^3$		Δu (5)
		(3)	(4)	
0.1 N HCl — 0.5 N glycine.....	3.02	7.67	5.4	2.37
0.02 N NaAc — 0.2 N HAc — 0.08 N NaCl.....	3.62	5.43		
0.02 N NaAc — 0.1 N HAc — 0.08 N NaCl.....	3.91	4.14		
0.1 N NaAc — 0.1 N HAc.....	4.64	0.36		
0.1 N NaAc — 0.05 N HAc.....	4.90	-0.96		
0.1 N NaAc — 0.01 N HAc.....	5.64	-2.68	-4.68	2.0
0.02 N NaCac — 0.004 N HCac — 0.08 N NaCl....	6.76	-4.24	-5.71	-1.47
0.02 N NaV — 0.02 N HV — 0.08 N NaCl.....	7.84	-6.12	-6.64	-0.62
0.1 N NaV — 0.02 N HV.....	8.62	-6.64	-6.90	-0.26
0.1 N NaV — 0.005 N HV.....	9.22	-7.54	-7.53	

Ac = acetate.

Cac = cacodylate.

V = diethylbarbiturate.

TABLE III
Precipitin Reactions in Sera of Rabbits Immunized with Native and Oxidized Bovine Serum Albumin

Serum of rabbit injected with	Test antigen used	Final dilution of test antigen			
		1:2,000	1:10,000	1:50,000	1:250,000
A	A	++++	++++	+++	+
A ₂₄	A ₂₄	0	0	+	±
	A	0	0	+	±
A ₇₂	A ₇₂	0	0	0	0
	A	0	0	0	0

A = native bovine serum albumin.

A₂₄ = bovine albumin oxidized for 24 hours with 0.03 M LiIO₄.A₇₂ = bovine albumin oxidized for 72 hours with 0.03 M LiIO₄.

++++ = complete precipitation with clear supernate.

+ = very slight precipitation.

0 = no precipitation.

of a week and again injected during 6 days, developed antisera which precipitated strongly the homologous protein. Groups of animals which had received a similar course of immunization with the protein treated with lithium periodate over a 24 and a 72 hour period gave, in the first instance, antisera which were very weak indeed. Those which had received the protein oxidized for 72

hours had no precipitins whatsoever (Table III), nor did they give a detectable immune response even after prolonged injection of the oxidized protein. These observations are contrary to those made with Type III pneumococcus immune globulin (1), where it was found that contact with lithium periodate for 24 hours did not destroy antigenicity.

TABLE IV

Precipitin Reactions of Oxidized Bovine Serum Albumin in Serum of a Rabbit Immunized with Native Albumin

Test antigen used	Final dilution of test antigen			
	1:2,000	1:10,000	1:50,000	1:250,000
A	++++	++++	+++	+
A ₂₄	++++	+++	++	+
A ₄₈	++	+±	+	±
A ₇₂	++	+±	+	0

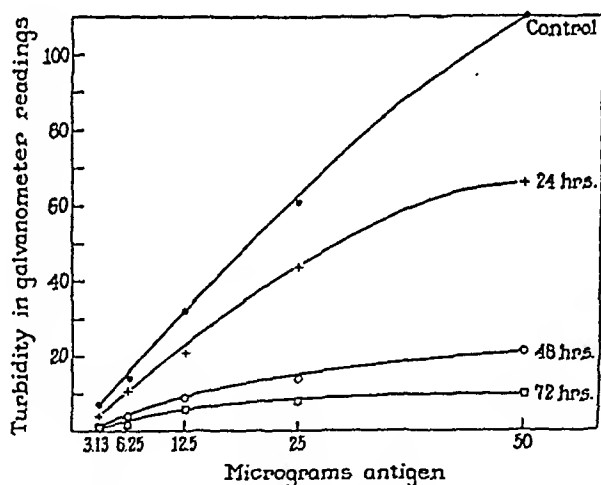


FIG. 3. Turbidimetric precipitin reaction of oxidized bovine albumin in native albumin antiserum.

Not only did the oxidized albumin fail to elicit homologous antibodies in rabbits, as can be seen in Table III, but there were no antibodies present in these sera capable of precipitating the native unaltered protein. This would indicate that no intact native protein molecules remain after the albumin has been treated with lithium periodate. It is of considerable interest, however, to note that the treated albumin is still capable of precipitating some of the antibodies in the sera of rabbits immunized with native serum albumin (Table

IV). When a quantitative turbidimetric estimation (11) of these reactions was made, it was observed (Fig. 3) that the intensity of the reaction diminished the longer the albumin fraction remained in contact with the reagent. From the results of these experiments it appears justified to conclude that the ability of serum albumin to incite antibody formation in rabbits is destroyed when the protein is subjected to the action of lithium periodate. The reason for this probably resides in the oxidative changes which take place in certain of the amino acids; these changes are not so profound, however, as to impair the ability of the altered protein to combine with the antibodies specifically directed toward the intact native protein molecule.

DISCUSSION

Periodic acid is not a benign reagent but a vigorous oxidant capable of bringing about extensive chemical changes in many types of organic compounds (12). Certain monosaccharides in acid solution are rapidly and completely broken down by the reagent; polysaccharides of appropriate structure suffer a severance of the component monosaccharide molecules with the formation of polyaldehydes and other ill defined products of oxidation. In addition, amino acids of certain types are readily oxidized by periodic acid. It is not surprising, therefore, that proteins may also undergo chemical alteration when brought in contact with the reagent.

That bovine serum albumin suffers irreparable damage on prolonged contact with lithium periodate is apparent from the results presented above. The chemical changes involve a slight loss in total nitrogen, a change in specific optical rotation, and an alteration or possible destruction of all or part of certain amino acids. The total sulfur content of the protein is somewhat diminished, yet the cystine, cysteine, and tyrosine content, the measure of which is based upon color development which in turn is dependent upon a specific chemical configuration, are considerably diminished. The tryptophane appears to be completely destroyed. That these chemical alterations are progressive and are a function of time is apparent from the gradual changes which occur in the absorption spectrum, and in electrophoretic behavior of the protein. Yet these changes are not so great as to cause a loss of properties which would no longer permit the material to be classified as protein. Solutions of the product of oxidation are still precipitated by high concentrations of salts; they give positive biuret tests, are precipitable by trichloroacetic acid, and are hydrolyzed by crystalline trypsin. In some respects the oxidized material resembles a denatured protein, —in absorption spectrum, in solubility in high concentration of salts, and in precipitability at acid pH values. Unlike denatured protein, the oxidized material is incapable of eliciting antibodies in rabbits. This loss of function is believed to be associated with alterations in the aromatic amino acid content, and in this respect the material is analogous to gelatin (13)

which contains neither tyrosine nor tryptophane, and is not antigenic. That the oxidized protein is still specifically precipitated by antibodies to native albumin, can best be explained on the basis that not all those groups of the protein which are responsible for antigen-antibody combination have been destroyed, even after prolonged contact with the reagent.

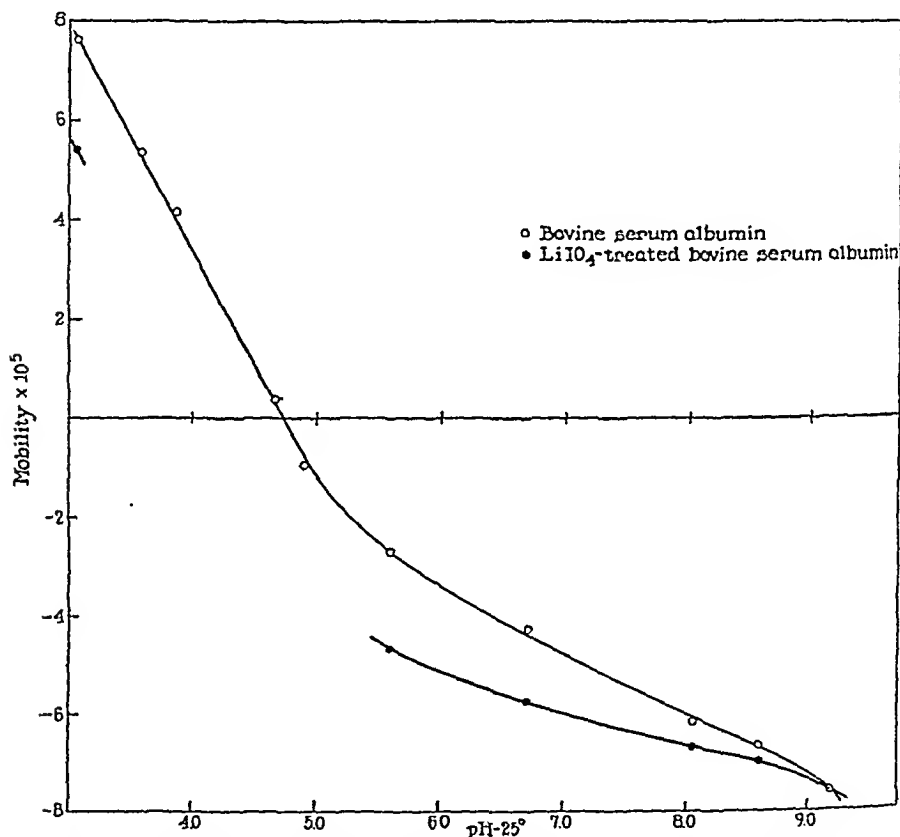


FIG. 4. Mobilities of normal and oxidized bovine serum albumin as a function of pH.

Because of the limited solubility of the oxidized protein, the mobility measurements recorded in this paper were necessarily confined to pH 3.0 and to the pH range of 5.6 to 9.2. A determination of the isoelectric point of the periodate-treated protein was therefore not possible. If the mobility data presented in Table II are plotted as ordinate against pH as abscissae, Fig. 4, it is apparent that the oxidized bovine serum albumin has a higher negative net charge than the normal bovine serum albumin at any given pH. This would indicate that the isoelectric point of the oxidized protein is at a pH more acid than that of

the native albumin and might possibly be explained by the destruction of the imidazole ring of the histidine molecule. The results of this report, however, do not permit of any extensive discussion concerning the groups which may have been altered during the oxidative process and which contribute to the electrophoretic behavior of this protein.

SUMMARY

A study of the chemical, physicochemical, and immunological changes in bovine serum albumin, brought about by oxidation with lithium periodate, has been made. It has been shown that destruction of certain amino acids occurs, that a change in the absorption spectrum takes place, and that the electrophoretic behavior of the protein is altered. Prolonged contact of bovine albumin with lithium periodate destroys its ability to incite antibodies in experimental animals.

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THE MECHANISM OF ACTIVE CEREBRAL IMMUNITY TO EQUINE ENCEPHALOMYELITIS VIRUS

I. INFLUENCE OF THE RATE OF VIRAL MULTIPLICATION

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It has been found (1) that mice vaccinated with inactivated Western equine encephalomyelitis (W.E.E.) virus were less resistant to intracerebral challenge doses of the "R.I." strain than to comparable doses of other strains of W.E.E. virus. This observation could not be ascribed to antigenic or serologic differences between the R.I. and other strains, nor to differences in immunizing potency, since immunization with the R.I. strain protected mice more effectively against heterologous strains of W.E.E. virus than against the homologous strain. The latter had been subjected, over a period of many years, to many brain-to-brain passages in mice, and in the course of this treatment its lethal titer had increased and the survival time of inoculated mice was considerably shortened. It seemed most likely that the difficulty in protecting mice against the R.I. strain was connected with its ability to kill mice more rapidly. The hope seemed warranted that further comparative studies on the properties of this and one of the other strains would lead to a better understanding of factors involved in the mechanism of immunity to neurotropic viruses. The present paper deals in the main with studies on the comparative growth rates in the mouse brain of the R.I. and another strain of W.E.E. virus. In addition, further experiments will be described which illustrate the effect of strain differences on the reaction of immunized animals to intracerebral inoculation. The host factors which are responsible for variations in the response will be discussed in a subsequent paper (2).

Materials and Methods

Virus Strains.—The 2 strains of W.E.E. virus used were the R.I. strain and the Kelser strain previously described by Olitsky, Morgan, and Schlesinger (1). They differed in the manner already mentioned; *i.e.*, the R.I. strain had a higher lethal titer and, after intracerebral inoculation of comparable amounts, killed mice about twice as rapidly as the Kelser strain. The R.I. strain has been carried through many brain-to-brain passages in mice since 1933, while the Kelser strain has been subjected to only a few such transfers. The exact number of passages has not been recorded for either strain.

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chloroformed after 40 hours when they began to show signs of encephalitis. Under deep anesthesia, the thoracic cavity was opened, the heart cut through, and the blood was drained onto absorbent cotton covered by gauze. The brains were removed, and excessive blood was washed off with saline. 4 ml. of cold saline was added per brain to make a 10 per cent suspension which was homogenized for 1 minute in a chilled Waring blender. It was then filtered through several layers of gauze, and finally formalin was added to a final concentration of 0.3 per cent. The vaccine was shaken daily and kept in a refrigerator. It was used when proved non-infectious by intracerebral test in mice.

As a rule, immunization consisted of 6 intraperitoneal doses given in 2 courses of 3 daily injections 1 week apart. The stock vaccine was diluted in saline and the inoculum was 0.3 ml. The total amount of vaccine given is stated in the text in terms of equivalent amounts of undiluted vaccine. Challenge inoculations were given 2 weeks after the first dose of vaccine.

Mice.—Albino Swiss mice, weighing 7 to 10 gm., were obtained from 2 dealers. There was no difference between them in response to any of the procedures used.

EXPERIMENTAL

Comparative Rates of Multiplication of the R.I. and Kelser Strains of W.E.E. Virus

(a) *The Initial Yield of Virus in Relation to the Amount Inoculated*.—A finding common to all the present experiments was an initial decrease in the amount of virus recoverable from infected brains. Inoculation of a volume of 0.03 ml. into a brain weighing 0.4 gm. would result in 13.3-fold dilution. Hence, after inoculation of 10^6 LD₅₀, the expected yield prior to viral multiplication would be $10^{(n-1.12)}$ LD₅₀ per 0.03 gm. of brain tissue. Actually, the mean yield at 1 hour after inoculation was only 3.5 per cent of the expected recovery in the case of the R.I. strain and 10.2 per cent in that of the Kelser strain. The disappearance from the brain of 90 to 96 per cent of the inoculum was independent of the amount of virus given, as shown in Tables III and IV. The significance of this loss is not clearly understood,¹ and for the purpose of studying the viral growth rate the extent of the loss is important mainly because it establishes the true starting point of multiplication.

(b) *Correlation between Rate of Virus Increase and Course of Disease in Mice*.²—Of 45 mice inoculated intracerebrally with $10^{2.6}$ LD₅₀ of the R.I. strain, 30 were sacrificed in pairs at 2 to 4 hour intervals. Definite signs of disease, i.e. spontaneous convulsive seizures or continuous circling, were first seen 38 hours after inoculation. All remaining mice died after 42 to 48 hours. 64 mice received intracerebrally $10^{3.4}$ LD₅₀ of the Kelser strain, and of these 48 were sacrificed in pairs at 4 hour intervals. Here, definite encephalitic signs began 74 hours after inoculation and the survival time of the remaining mice varied from 3 to 7 days with an average of 3.7 days.

¹ Losses of similar magnitude were encountered when *E. coli* phages T1 or T7 were inoculated intracerebrally in mice.

² The samples of the 2 strains of virus used in this test have not been included in Tables I and II.

The rates of multiplication of the 2 strains are presented in Fig. 1. The titer of the R. I. strain rose logarithmically up to 32 hours after inoculation. By that time, its increase was 1,000 times greater than that of the Kelser strain. The R.I. strain reached its maximum titer about 6 hours before onset of definite encephalitic signs. The growth rate of the Kelser strain appeared to slow

TABLE III

W.E.E. Virus, R.I. Strain: Difference between Expected and Actual Yield of Virus from Mouse Brains 1 Hour after Intracerebral Inoculation

Test No.	Inoculum (a)*	Yield at 1 hr.		Difference (b) - (c)*	Deviation from mean difference (d)	(d)†
		Expected = (a) - 1.12 (b)*	Actual (c)*			
1	3.2	2.08	1.12	0.96	-0.49	0.240
2	3.7	2.58	0.70	1.88	+0.43	0.185
3	3.7	2.58	0.95	1.63	+0.18	0.032
4	4.2	3.08	1.5	1.58	+0.13	0.017
5	4.2	3.08	1.0	2.08	+0.63	0.397
6	4.2	3.08	1.5	1.58	+0.13	0.017
7	4.2	3.08	1.7	1.38	-0.07	0.049
8	5.2	4.08	3.0	1.08	-0.37	0.137
9	5.2	4.08	2.73	1.35	-0.10	0.010
10	7.2	6.08	4.75	1.33	-0.12	0.014
11	7.2	6.08	4.88	1.20	-0.25	0.063
12	8.2	7.08	5.95	1.13	-0.32	0.102
13	8.2	7.08	5.37	1.71	+0.26	0.068
Total (Σ).....	68.6	54.04	35.15	18.89	3.48	1.331
Mean $\frac{\Sigma}{N}$	5.277	4.157	2.704	1.453	0.268	—

$$\text{Standard deviation } (\sigma) = \sqrt{\frac{\Sigma(d^2)}{N-1}} = \sqrt{\frac{1.331}{12}} = 0.333$$

* All figures given as log LD₅₀.

down beginning about 36 hours after inoculation* and came to a standstill after about 62 hours or 12 hours before onset of definite encephalitic signs.

Thus, the difference between the 2 strains with regard to incubation and survival periods reflected a difference of similar magnitude between their rates of multiplication.

(c) *Latent Phase Preceding Multiplication of the R.I. and the Kelser Strains.*—As is shown in Fig. 2, no viral growth was demonstrated for about 3 hours in the brains of mice inoculated with 10^{3.7} LD₅₀ of the R.I. strain. In the case of the Kelser strain, after inoculation of 10^{4.5} LD₅₀, there was no significant multiplication for 5 hours (Fig. 3). After these latent periods, there was

growth at a fairly constant rate of the R.I. strain, while that of the Kelser strain appeared to come to a standstill between 7 and 10 hours after inoculation. Between 3 and 10 hours after inoculation, there was a 1,000-fold increase in titer of the R.I. strain, and only a 10-fold increase in that of the Kelser strain.

(d) *Influence of Variations in the Size of the Inoculum on the Rate of Viral Multiplication.*—After inoculation of various amounts of W.E.E. virus in excess of 100 LD₅₀ there was relatively little variation in the survival time

TABLE IV

W.E.E. Virus, Kelser Strain: Difference between Expected and Actual Yield of Virus from Mouse Brains 1 Hour after Intracerebral Inoculation

Test No.	Inoculum (a)*	Yield at 1 hr.		Difference (b) - (c)*	Deviation from mean difference (d)	(d) ²
		Expected = (a) × 1.12 (b)*	Actual (c)*			
1	2.5	1.38	<0.70	>0.68	>0.31	0.096
2	2.5	1.38	<0.50	>0.88	>0.11	0.012
3	4.0	2.88	2.50	0.38	-0.61	0.372
4	4.0	2.88	2.50	0.38	-0.61	0.372
5	4.0	2.88	1.25	1.63	+0.64	0.409
6	4.5	3.38	2.62	0.76	-0.23	0.053
7	4.5	3.38	2.38	1.00	+0.01	0.0001
8	4.5	3.38	2.50	0.88	-0.11	0.012
9	4.5	3.38	1.66	1.72	+0.73	0.533
10	4.5	3.38	1.91	1.47	+0.48	0.230
11	6.5	5.38	4.12	1.26	+0.27	0.073
12	6.5	5.38	4.50	0.88	-0.11	0.012
Total (Σ).....	52.5	39.06	27.14	11.92	4.22	2.173
Mean $\frac{\Sigma}{N}$	4.375	3.255	2.261	0.993	0.352	—

$$\text{Standard deviation } (\sigma) = \sqrt{\frac{\Sigma(d^2)}{N-1}} = \sqrt{\frac{2.173}{11}} = 0.444$$

* All figures given as log LD₅₀.

of mice (1). This similarity in the course of the disease is matched by the observation that the growth curves after inoculation of different amounts tended to converge so that the maximum titer was reached at about the same time regardless of the size of the inoculum. This is illustrated for the 2 strains in Figs. 4 and 5. It will also be seen that after inoculation of relatively small amounts of the R.I. strain (see Fig. 4), there was a tendency for the growth rates to parallel each other.

On the basis of these findings regarding the growth rates of the 2 strains,

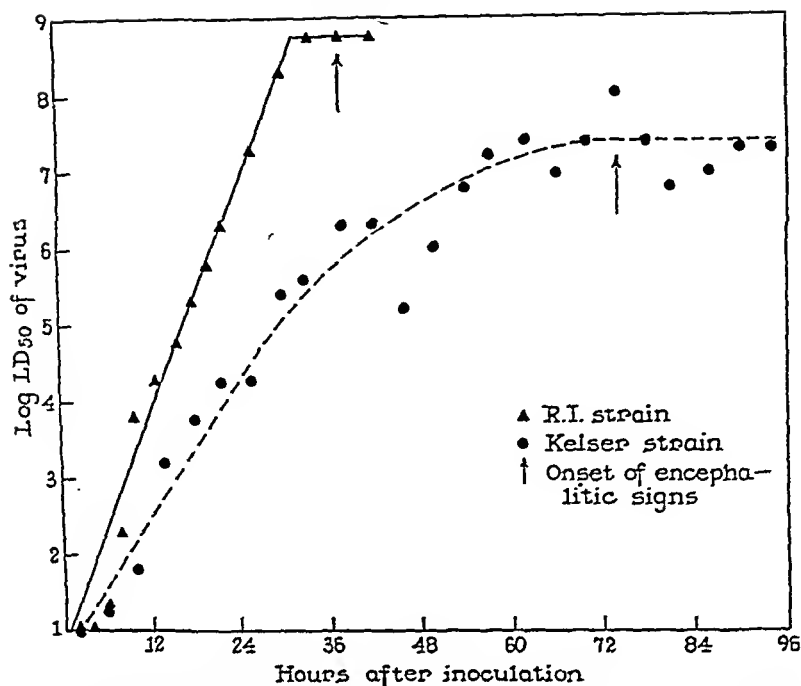


FIG. 1. Comparative rates of multiplication of R.I. and Kelser strains of W.E.E. virus in brains of normal mice.

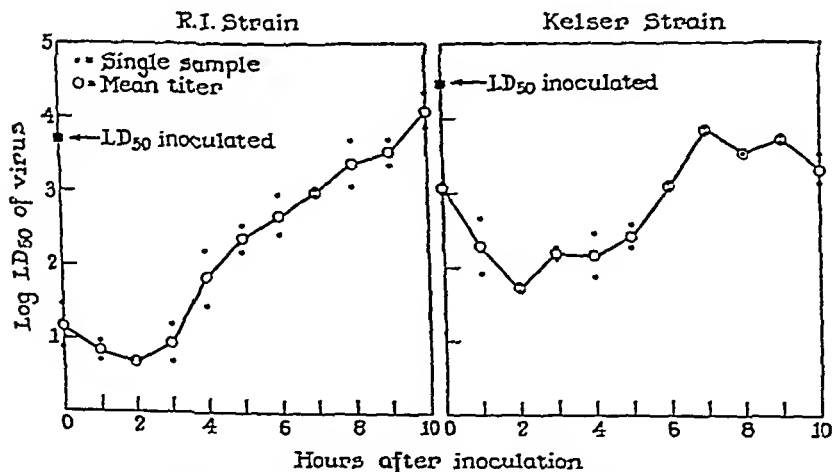


FIG. 2

FIG. 3

FIGS. 2 and 3. Rate of multiplication of W.E.E. virus in brains of mice after intracerebral inoculation.

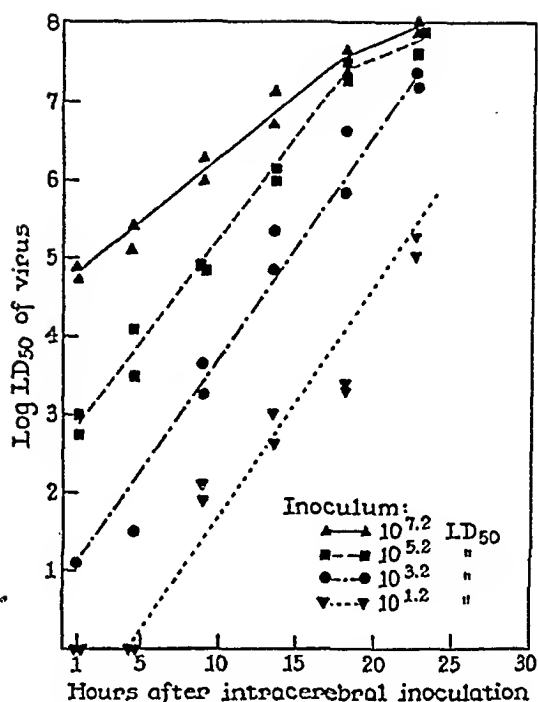


FIG. 4. Rates of multiplication of W.E.E. virus, R.I. strain, after intracerebral inoculation of different amounts.

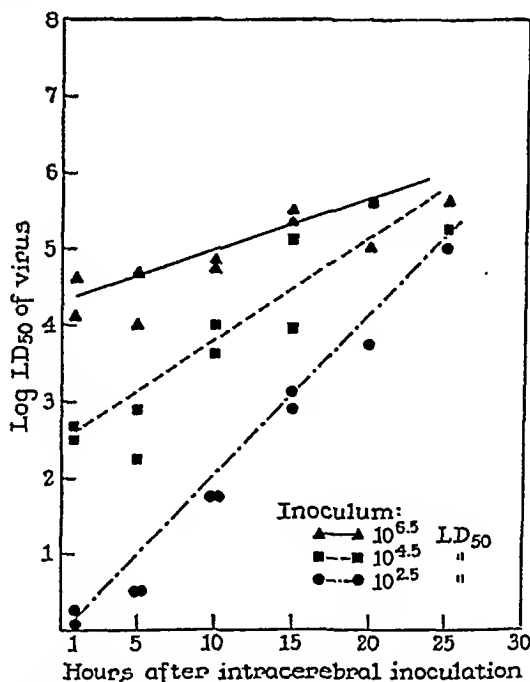


FIG. 5. Rates of multiplication of W.E.E. virus, Kelser strain, passage 3, after intracerebral inoculation of different amounts.

the difference in response to them of immunized mice was reexamined. The primary purpose of the experiments to be described in the following sections was to define this difference quantitatively and to establish a baseline for an investigation into its immediate cause.

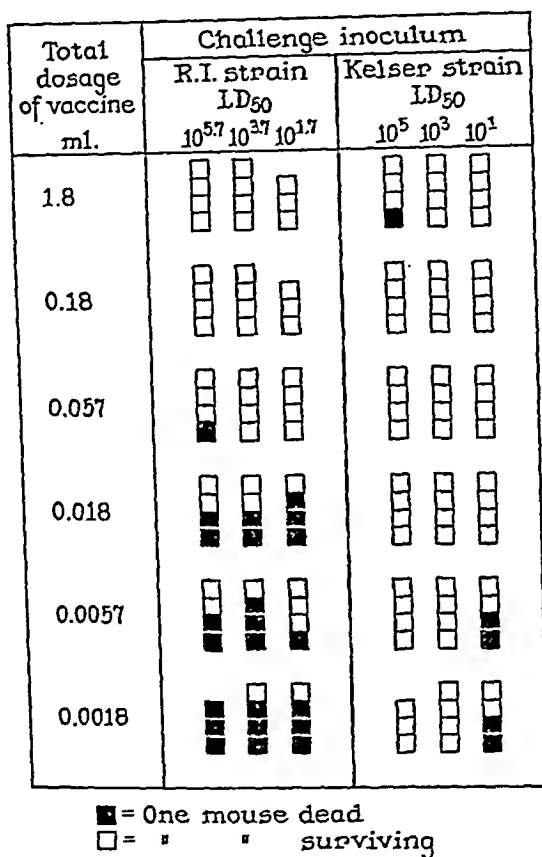


FIG. 6. Comparative degrees of resistance of immunized mice to intracerebral challenge doses of two strains of W.E.E. virus.

The Response of Immunized Mice to Intracerebral Challenge Doses of the R.I. and Kelser Strains

Fig. 6 presents the results of an experiment in which mice immunized with different amounts of vaccine were challenged by the intracerebral route with comparable graded amounts of the R.I. or the Kelser strain of W.E.E. virus. It is clear that after immunization with relatively small doses of vaccine (less than 0.057 ml.) there was a much greater degree of resistance to the Kelser

than to the R.I. strain. This difference in response was masked when a larger dosage of vaccine was used.³

It is noteworthy that four of eight mice immunized with the smallest amounts of vaccine (0.0057 and 0.0018 ml.) succumbed to a challenge dose of 10 LD₅₀ of the Kelser strain while all the mice challenged with 10³ or 10⁵ LD₅₀ survived. This "paradoxical" type of response was found to be characteristic of mice with low degree of immunity and will be discussed in a subsequent paper (2).

TABLE V
Comparison of Typical Titrations in Mice of Three Representative Samples of W.E.E. Virus

Sample of virus	Dilution of virus	Day of death		Total mortality	Log LD ₅₀
		Individual mice	Average		
R.I. strain	10 ⁻⁷	2, 2, 2, 2	2.0	4/4	9.2
	10 ⁻⁸	3, 3, 4, 5	3.75	4/4	
	10 ⁻⁹	3, 3, 0, 0	3.0	2/4	
	10 ⁻¹⁰	4, 0, 0, 0	(4)	1/4	
Kelser strain passage 3	10 ⁻¹	6, 7, 7, 7, 8	7.0	5/5	>7.4
	10 ⁻²	6, 7, 7, 7, 9	7.2	5/5	
	10 ⁻³	6, 6, 6, 7, 9	6.8	5/5	
	10 ⁻⁴	5, 7, 8, 8, 9	7.4	5/5	
	10 ⁻⁵	6, 6, 7, 8, 9	7.2	5/5	
	10 ⁻⁶	5, 6, 6, 7, 8	6.4	5/5	
	10 ⁻⁷	5, 6, 8, 8, 0	6.75	4/5	
	10 ⁻⁸	3, 3, 3, 3, 7	3.8	5/5	
Kelser strain passage 40	10 ⁻⁷	3, 3, 3, 4, 6	3.8	5/5	7.6
	10 ⁻⁸	4, 0, 0, 0, 0	(4)	1/5	
	10 ⁻⁹	0, 0, 0, 0, 0	—	0/5	

Effect of Continued Brain-to-Brain Passages on the Behavior of the Kelser Strain

In an effort to show that the distinct properties of the R.I. strain had resulted from its continued propagation in the mouse brain, the slower Kelser strain was subjected to a rapid succession of brain-to-brain passages in mice.

After the virus had undergone a total of 40 passages in this laboratory,

³ In earlier experiments (1), the difference between strains was demonstrable even in mice immunized with relatively large doses of vaccine. At that time, vaccines had been prepared from infected chick embryo and had been centrifuged. They were less potent than the ones used more recently. Studies on St. Louis and Japanese B encephalitis vaccines have shown that centrifugation may result in considerable loss of antigenic potency (6). The present studies, carried out with crude vaccines prepared from infected mouse brain, confirm the report of Ruchman (7) to the effect that there is no difficulty in effectively immunizing mice against the R.I. strain.

TABLE VI

Comparative Neutralization Test with Mouse Immune Serum against the R.I. Strain and Two Variants of the Kelser Strain of W.E.E. Virus

Log final dilution of serum	R.I. strain				Kelser passage 3				Kelser passage 40			
	LD ₅₀ in mixture			Neutralization index*	LD ₅₀ in mixture			Neutralization index	LD ₅₀ in mixture			Neutralization index
	10 ^{2.2}	10 ^{2.3}	10 ^{2.4}		10 ²	10 ^{2.1}	10 ^{2.0}		10 ^{2.2}	10 ^{2.3}	10 ^{2.4}	
0.8	1/4†	0/4	0/4	>3.53	1/4	0/4	0/4	>3.33	2/4	0/4	0/4	3.2
1.8	4/4	4/4	0/4	1.7	3/4	1/4	1/4	2.33	4/4	0/4	1/4	2.58
2.3	4/4	3/4	0/4	1.87	4/4	4/4	1/4	1.33	4/4	3/4	0/4	1.87
2.8	4/4	3/4	2/4	1.4	3/3	3/3	1/4	1.33	4/4	4/4	2/4	1.2
3.3	4/4	3/4	2/4	1.4	4/4	3/4	2/4	1.2	4/4	4/4	0/4	1.7
3.8	4/4	4/4	2/4	1.2	4/4	4/4	3/4	<0.7	4/4	4/4	4/4	<0.7
Neutralizing titer§	1.13	1.74	3.3	—	1.3	2.04	3.1	—	0.8	1.83	3.36	—

* Neutralization index, log LD₅₀ of virus neutralized by indicated dilution of serum.

† Numerator, number of mice dead. Denominator, number inoculated.

§ Neutralizing titer, log of estimated highest dilution of serum which would protect 50 per cent of the mice in mixture with indicated amount of virus.

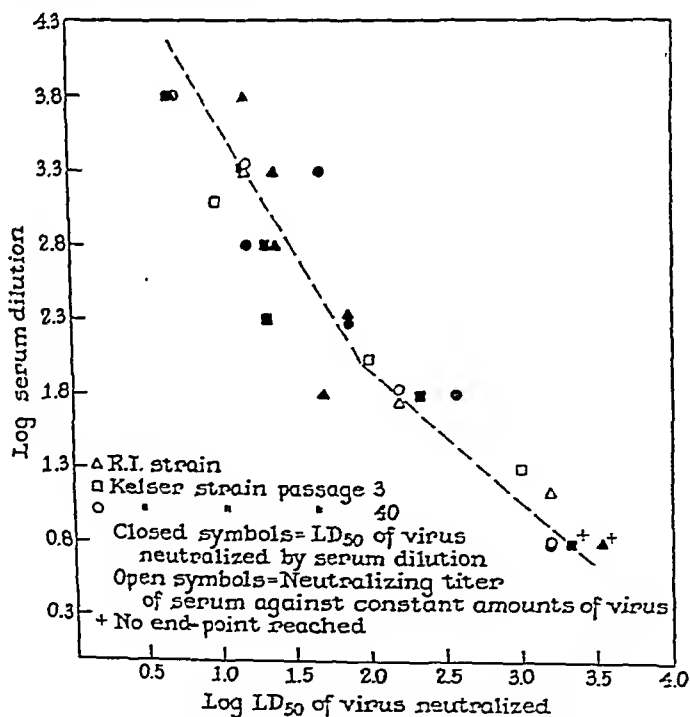


FIG. 7. Neutralization of W.E.E. virus by hyperimmune mouse serum.

convulsive seizures were regularly seen in all mice 2 days after inoculation of 10^{-2} diluted brain tissue. In Table V, typical titrations of the R.I. strain and of 3rd and 40th passage Kelser virus are presented. Although the titer of the 40th passage virus was still relatively low, the average survival period of mice infected with it had so decreased as to approach that of mice infected with the R.I. strain.

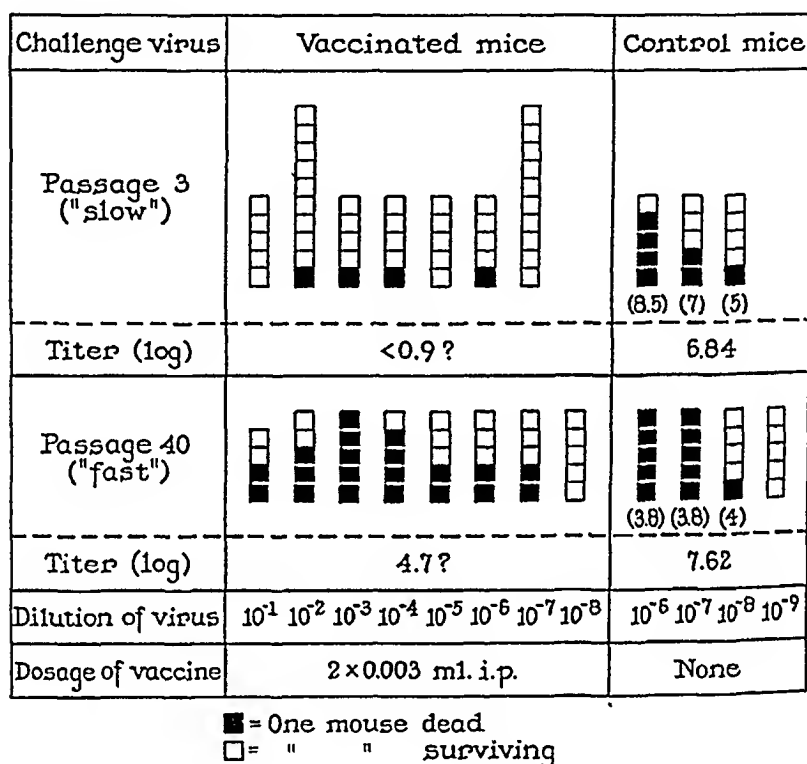


FIG. 8. Comparative degrees of resistance of immunized mice to intracerebral challenge doses of "slow" and "fast" variants of the Kelser strain.

Numbers in parentheses indicate the average survival periods in days.

The same three virus preparations were employed in a neutralization test for the purpose of establishing their serological identity.

Serial 0.5 log-fold dilutions of a W.E.E. R.I. immune mouse serum were mixed with approximately 10, 100, and 1,000 LD_{50} of each of the 3 virus samples. After 2 hours' incubation in the 37°C. water bath, the mixtures were injected intracerebrally into mice. The results of this test are given in Table VI. The data are summarized in Fig. 7 which shows that the 3 samples were neutralized equally well by the serum. Thus, no measurable serological changes

resulted from continued propagation of either strain of W.E.E. virus in the mouse brain.

It remained to be seen whether the "fast" derivative of the Kelser strain differed from its parent strain when used as challenge inoculum in immunized mice. After immunization with 0.006 ml. of vaccine, mice were challenged intracerebrally with graded doses of passage 3 or passage 40 samples of the Kelser strain. The outcome of this test is presented in Fig. 8. After 37 additional passages, the Kelser strain behaved like the R.I. strain in that mice vaccinated to an extent such that they resisted almost without exception even maximal amounts of the "slow" strain were only slightly protected against its "fast" derivative. In the latter group, deaths were scattered over the entire range of virus dilutions. This illustrates the inability to express intermediate degrees of immunity in mathematical terms. With the small number of animals employed, it appeared that the chance of survival was hardly greater after challenge with 4 than with 4 million LD₅₀.

DISCUSSION

The data presented in this paper have confirmed the fact that continued propagation of W.E.E. virus in the mouse brain may yield a viral variant with increased rapidity of action. The greater speed with which such adapted virus killed mice was paralleled by a corresponding increase in its rate of multiplication in the brain. This was associated with a shortened initial latent period during which there was no measurable increase in viral titer.

In mouse brains infected with W.E.E. virus, the titer of recoverable virus was 90 to 96.5 per cent lower than the theoretical yield, and it was at this reduced level that the virus maintained itself for about 3 hours in the case of the fast R.I. strain and for about 5 hours in that of the slow Kelser strain. The initial drop in detectable virus may have been due simply to leakage into other tissues, or it may have been caused by adsorption of a proportion of the inoculum onto host cells with resulting loss of infectivity. The observation that losses of similar magnitude followed the intracerebral inoculation of bacteriophage does not necessarily rule out the latter possibility. With the nature of this initial loss unexplained, it remains doubtful whether the latent period represented a temporary equilibrium between rate of disappearance from the brain and rate of virus increase, or whether it was due to a latent phase in the intracellular growth cycle comparable to the "constant periods" described for bacteriophage (8) and influenza viruses (9). A more conclusive interpretation of the growth experiments is difficult because of the inability to separate unadsorbed or newly liberated virus from infected host cells.

It is interesting, however, that after inoculation of various large amounts of either strain of W.E.E. virus, the rates of multiplication tended to converge, while after inoculation of the R.I. strain in amounts closer to the mini-

mal lethal dose they tended to parallel each other. This latter finding suggests that of the small amount of virus all was utilized in infecting host cells. Convergence in the higher range may indicate that an increasingly high proportion of the seed virus was in excess of the amount immediately utilized to initiate infection. It will be shown in the following paper (2) that this postulated excess may have an important function as free antigen in the mechanism of immunity in vaccinated animals.

The growth experiments here reported, while of some obvious interest in relation to the broader problems of virus-host relationship and viral adaptation, have their chief significance in connection with the difference in response of immunized animals to intracerebral challenge doses of the "fast" and the "slow" variants of W.E.E. virus. This difference was so striking that one may suspect serological heterogeneity. That minor serological changes may occur upon continued propagation of viruses in certain hosts has been suggested by studies on influenza virus (10). Careful investigation, however, has failed previously (1) and again in the present work to reveal detectable serological changes resulting from adaptation of W.E.E. virus to the mouse brain. The mechanism which enables vaccinated animals to survive infection with a "slow" strain but not with a "fast" derivative from it will be described in the following paper (2).

The different response of vaccinated animals to 2 serologically indistinguishable variants of the same virus may have some practical significance. It is conceivable that active immunity tests with variants of other viruses having similar differences in growth rates may have created the impression that they represented strains of different immunological types. This possibility should be considered especially in the case of poliomyelitis virus where immunological differentiation of strains has often been based on active immunity rather than on neutralization tests.

Similarly, certain standard potency tests to which vaccines prepared for medical and veterinary use are subjected involve intracerebral challenge inoculations in vaccinated mice. Habel and Wright (11) have recently recognized that various strains of rabies virus when used for challenge in such tests may cause wide variations in the results obtained, and on this basis they have recommended the use of aliquots of a single sample of standard challenge virus in all laboratories.

The results and conclusions presented in this and in the following paper (2) for W.E.E. virus may apply equally to similar variations encountered with other viruses.

SUMMARY

Continued serial brain-to-brain passage of strains of W.E.E. virus in mice has yielded variants which kill mice with increased rapidity. Their rate of

multiplication in the mouse brain has been found to be correspondingly increased.

At 1 hour after intracerebral inoculation of various amounts of W.E.E. virus, only 3.5 to 10 per cent of the expected amount of virus was recovered from the infected brains.

In infected mouse brains, the period of active viral multiplication was preceded by a latent phase which lasted a considerably shorter time in the case of a "fast" than in that of a "slow" variant.

In brains inoculated with various amounts in excess of minimal lethal doses the rates of multiplication tended to converge with the result that the maximum titer was reached after about the same period of time. After inoculation of smaller amounts, the rates of viral multiplication tended to parallel each other.

Vaccinated mice may be fully resistant to maximal intracerebral doses of a slowly multiplying strain while they are not at all or only partly protected against a rapidly multiplying one derived from it. This difference is demonstrable even though fast and slow variants are, as far as can be tested, serologically identical. The difference in response may be masked if animals are immunized with relatively large doses of vaccine.

The bearing of these findings on certain practical problems has been pointed out.

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THE MECHANISM OF ACTIVE CEREBRAL IMMUNITY TO EQUINE ENCEPHALOMYELITIS VIRUS

II. THE LOCAL ANTIGENIC BOOSTER EFFECT OF THE CHALLENGE INOCULUM

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In earlier studies on the mechanism of immunity to the viruses of Eastern (E.E.E.) or Western equine encephalomyelitis (W.E.E.), it was possible, under certain experimental conditions, to demonstrate a quantitative correlation between degree of cerebral resistance of vaccinated mice and titer of neutralizing antibody in their serum (1). The high serum titer generally required to insure resistance to intracerebral challenge was believed to be due to penetration of only a small fraction of serum antibody into the central nervous system since the serum/ brain tissue/ spinal fluid ratio for antibody titers in vaccinated rabbits was of the order of 300/3/1 (2).

However, when the response of immunized rabbits or guinea pigs to an intracerebral inoculum was studied in some detail (3), it was found that the serum/ brain antibody ratio was not unalterably fixed. The inoculation was followed first by a febrile response closely resembling that of non-vaccinated animals. In some instances, there were also transitory signs of damage to the central nervous system. It was possible to isolate the virus from the brains of a few guinea pigs during this abortive attack, but attempts to demonstrate viral multiplication failed repeatedly. In one experiment, the rise and fall in neutralizing titers of sera and brains was followed after challenge inoculation, and it was found that at 1 week the brain titer had risen so high that the serum/brain ratio had been reduced from about 100/1 to 1/1 and 10/1, respectively, in the two animals tested.

It seemed possible that such high neutralizing titers of brain tissue, if reproducible, might be of significance in relation to the survival of immune animals. This was suggested, in particular, by the finding (4) that the degree of resistance of vaccinated mice to W.E.E. virus depended on the rate of multiplication of the strain used as challenge inoculum. This difference in response indicated that the concentration of antibody present *at the time of challenge* was certainly not the sole factor determining the fate of the immune animal. It was likely that the intracerebral inoculum served as an antigenic booster and that the effectiveness of the resulting antibody response depended on the rate of viral multiplication. Experiments in this direction were carried out in mice because it was desirable to use large numbers of animals, and because the growth rates

of viral variants were being studied in that host (4). This paper will deal with the following aspects: (a) the fate of a "fast" and a "slow" virus strain in the immune host; (b) the antigenic booster effect of the challenge inoculum; (c) the nature of the neutralizing substance found in brain tissue.

Materials and Methods

The R.I. and the Kelser strains of W.E.E. virus were used as representing "fast" and "slow" variants of the virus. Their characteristics and most of the other materials and methods used have already been described (4).

Serological Tests.—Test material for serological study was obtained as follows: Mice were exsanguinated from the heart, and then their heads were perfused slowly with at least 10 ml. of saline. The efficacy of perfusion was tested by suspending homogenized perfused normal brain tissue in 2.2 parts of distilled water (1:3.2) and then clarifying the suspension in the cold for 30 minutes at about 13,000 R.P.M. in an angle centrifuge.¹ The supernatant fluid was water-clear and colorless indicating the absence of detectable hemolysis.

Brains to be tested for antibody were kept frozen in lusteroid tubes. For test, they were ground up in 2.2 ml. per gm. of tissue of either saline containing 2 per cent of normal guinea pig serum previously heated at 56°C. for 30 minutes, or plain saline. The resulting suspensions were used either uncentrifuged or centrifuged for 30 minutes at 13,000 R.P.M. Neutralization tests with crude suspensions and supernatant fluids yielded identical results.

(a) *Technique and Interpretation of Neutralization Tests.*—Serial 0.5 log-fold (actually 3.2-fold) dilutions of serum or brain extract were mixed with equal parts of 2×10^{-8} diluted R.I. stock virus. The mixtures were held for 2 hours in the 37°C. water bath, and then 0.03 ml. was inoculated intracerebrally into groups of 4 to 6 mice per dilution. Control mixtures with normal mouse serum or brain extracts were always injected last.

The final concentration of virus in the mixtures (10^{-8}) represented $10^{1.2}$ LD₅₀/0.03 ml., since, as shown in the earlier paper (4), the mean titer of this virus was $10^{9.2}$ LD₅₀/0.03 gm. of brain tissue. In the period during which neutralization tests were carried out, a total of 36 control titrations were performed in which the 10^{-8} dilution was included. The number of mice used per test ranged from 4 to 10. Of 169 mice inoculated, only 4 survived; in 36 tests, there were only 3 in which 20 to 25 per cent of the mice survived (1 of 4, 1 of 5, 2 of 10, respectively). The odds, therefore, of 1 of 4, 1 of 5, or 1 of 6 mice surviving may be considered as 1 in 10, 1 in 8, 1 in 7 tests, respectively. The chance of 2 surviving in such groups would have been less than 1 in 100 or 1 in 1,000. Therefore, in neutralization tests the occurrence of 2 or more survivors in a group of mice may be considered as due to specific neutralization. However, deaths may be scattered among mice inoculated with "neutral" mixtures containing antibody dilutions over the entire effective range, the mortality rate not necessarily increasing with decreasing concentration of antibody. This variation in response of individual mice to a given inoculum makes difficult the determination of the actual limiting neutralizing dilution. If an adequate range of dilutions and enough animals are used, consistent and reproducible titers are obtained by applying the method of Reed and Muench (5) to calculate the cumulative 50 per cent survival end-point, or the estimated highest (final) dilution which, when injected together with $10^{1.2}$ LD₅₀ of virus, would protect 50 per cent of the mice (ND₅₀).

(b) *The Serum/Brain Ratio of Neutralizing Antibody in Immunized Mice.*—The methods just described can best be illustrated by an example which, at the same time, will show the "normal" serum/brain ratio in mice immunized by intraperitoneal inoculation of W.E.E. vaccine.

¹ A Sorval angle centrifuge with a 9 inch head was used.

Mice were immunized with a total of 0.17 ml. of vaccine, a relatively large dose. Two weeks after the first dose, they were exsanguinated and perfused, and their brains and sera were tested for neutralizing antibody.

TABLE I

Comparative Neutralizing Titers of Serum and Brain Tissue of Hyperimmunized Mice against $10^{1.2}$ LD₅₀ of W.E.E. Virus

Mouse No.	Test material	Rate of survival of mice							Log ND ₅₀ *	Ratio Serum Brain	
		Log final dilution of serum or brain in mixture								Log	Antilog
		0.8	1.3	1.8	2.3	2.8	3.3	3.8			
1	Serum	—	4/4†	3/3	3/4	4/4	2/4	—	>3.20§	>2.40	>252 1
	Brain	0/4	1/4	1/4	—	—	—	—	<0.80		
2	Serum	—	2/4	4/4	3/3	2/4	3/4	—	>2.95	>2.15	>142 1
	Brain	2/4	0/4	0/4	—	—	—	—	0.80		
3	Serum	—	3/4	4/4	4/4	3/4	2/4	—	>3.07	>2.12	>132 1
	Brain	3/4	0/4	0/4	—	—	—	—	0.95		
4	Serum	—	—	4/6	5/6	4/6	3/6	5/6	>3.30	>2.45	>282 1
	Brain	3/6	1/6	—	—	—	—	—	0.85		
5	Serum	—	—	3/6	5/6	6/6	3/6	1/6	3.10	2.25	178 1
	Brain	3/6	1/6	—	—	—	—	—	0.85		
Mean 1, 2, 3, 4, 5	Serum								>3.12	>2.27	>186 1
	Brain								<0.85		
1, 2, 3, 4, 5 pooled	Serum	—	—	—	5/5	5/5	4/5	0/5	3.49	2.29	195 1
	Brain	3/5	3/5	0/5	—	—	—	—	1.20		

Total dosage of vaccine (7 doses) = 0.17 ml. intraperitoneally.

* ND₅₀ = estimated highest dilution of test material which would protect 50 per cent of the mice.

† Fractions indicate number of mice surviving in numerator, number inoculated in denominator.

§ >3.20, no end-point; ND₅₀ = 3.20 or higher.

|| <0.80, no end-point; estimate of ND₅₀ based on assumption that with lower dilutions of antibody all mice would survive.

The results are given in Table I. While end-points were not obtained in all titrations of individual sera, the approximate mean serum/brain ratio for the 5 mice studied was similar to that obtained in a test with pooled materials from all 5 animals. A ratio of the order of 200/1 is in close accord with that described earlier (2) for rabbits vaccinated with W.E.E. virus. Similar values have also been found by Freund (6) for bacterial antibody in rabbits and by Fox (7) for yellow fever antibody in mice vaccinated by extraneural routes.² Since

² For the purpose of comparison, the ratios given by Fox (7) should be multiplied by 10, since 10 per cent brain suspensions served as starting materials in his titrations.

Freund found that this ratio is established shortly after either intravenous (6) or intracisternal (8) injection of antibody, one may assume that it expresses an equilibrium between plasma and brain tissue under normal physiological conditions. Accordingly, there were only a few exceptional tests in which the lowest dilutions of brain tissue from immunized mice neutralized even though the corresponding serum titer was less than 1:1,000.

(c) *Technique of Complement Fixation Tests.*—Brain suspensions serving as source of complement-fixing antibody were prepared as described above. They were heated for 20 minutes in the 60°C. water bath and then centrifuged for 30 minutes at 13,000 R.P.M. The resulting supernatant was water-clear and colorless. Sera were diluted 1:4 or higher in saline, heated at 60°C. for 20 minutes, and then also centrifuged for 30 minutes at 13,000 R.P.M.

Eastern and Western E.E. antigens were obtained from the Lederle Laboratories, Inc.

Tests were set up with 0.1 ml. of antigen, 0.1 ml. of serum or brain dilution, 0.2 ml. containing 2 hemolytic units of complement, and 0.2 ml. of sensitized sheep erythrocytes, and the technique used was that recommended by Casals (9). Titers have been expressed in terms of highest original dilutions of test material giving 2 plus or greater fixation.

EXPERIMENTAL

Fate of W.E.E. Virus after Intracerebral Inoculation in Immune Mice

For a study on the fate of W.E.E. virus in the brains of immunized animals, it was desirable to contrast the response to a "fast" variant with that to a "slow" one. Therefore, mice were vaccinated to a degree at which they were expected to resist the "slow" but not the "fast" inoculum. The dependence of demonstrability of this strain difference on the level of immunity has been described elsewhere (4).

Accordingly, mice received intraperitoneally a total of 0.018 ml. of vaccine. Four weeks after the first dose, 33 received intracerebrally $10^{5.7}$ LD₅₀ of the "fast" R.I. strain, and 39 $10^{5.5}$ LD₅₀ of the "slow" Kelser strain. Non-vaccinated mice of the same age served as controls. Of the mice challenged with the R.I. strain, 9 were sacrificed at various intervals up to the 3rd day. The remaining 24 died from 2 to 11 days (average 3.7 days) after inoculation. Of those challenged with the Kelser strain, 35 were sacrificed at various intervals up to 114 days after inoculation. None showed any signs of illness. Mice from the control groups were sacrificed at corresponding intervals as long as survivors were left.

Thus, vaccinated mice were resistant to $10^{5.5}$ LD₅₀ of the slow strain but not to $10^{5.7}$ LD₅₀ of the fast one.

All mice were perfused except those sacrificed before the 3rd day after challenge inoculation. The brains were used in tests for virus or antibody content.

The fate of the virus in the brain was followed in the manner already described (4) for experiments on viral multiplication. Brains of individual mice were titrated. The range of titers is given for the R.I. strain in Fig. 1, for the Kelser strain in Fig. 2. The curves for the 2 strains in the non-vaccinated groups were comparable with those described in detail elsewhere (4). In vaccinated mice, the rate of multiplication of the "fast" variant paralleled that in non-vaccinated controls but at a lower level (Fig. 1). Inasmuch as the brains at 3 hours and at 1 and 2 days were not perfused, it is possible that the

difference between normal and vaccinated mice may have been largely the result of *in vitro* neutralization.

On the other hand, in the vaccinated group challenged with the "slow" strain, there was a much more marked depression in the titer of demonstrable virus (Fig. 2). Nevertheless, multiplication apparently occurred, and in some

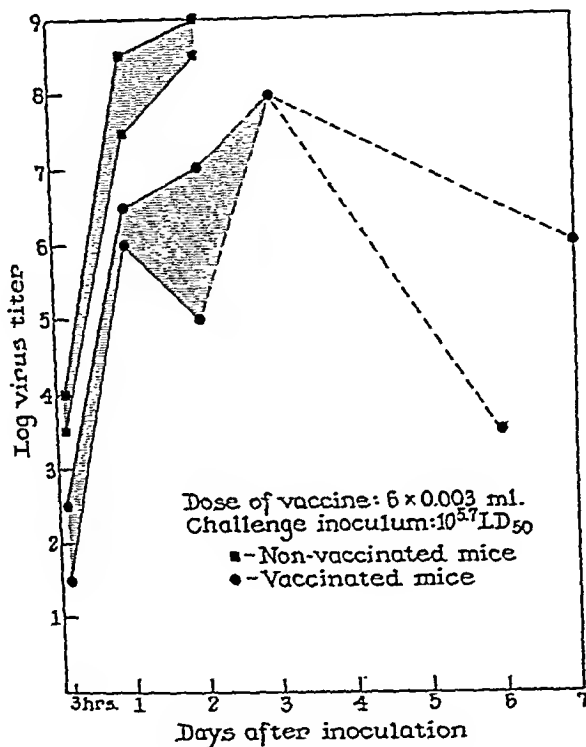


FIG. 1. Multiplication of W.E.E. virus, "fast" R.I. strain, in brains of normal and of vaccinated mice.

animals the virus maintained itself for 4 days at a level 100-fold higher than that found at 3 hours.

Because of the small number of specimens available in this test and because of the importance attached to the unequivocal demonstration of viral multiplication in immune animals, another experiment was done in which mice immunized with a total of 0.015 ml. of vaccine were challenged with $10^{5.5}$ LD₅₀ of the "slow" strain. Brains were harvested at 3 hours and at 2 days from 9 mice each. Of 30 additional mice, only one succumbed, and the group as a whole may be considered as resistant. At each interval, 3 pools of 3 brains each were titrated for virus. The results of these titrations are presented in Table II. It will be seen again that there was about a 100-fold increase in virus at 2 days in the brains of vaccinated, resistant mice.

Thus, immunity of a degree high enough to insure resistance to challenge did not preclude transitory multiplication of the challenge virus. However, while some factor arrested further multiplication of the "slow" strain, this factor failed to operate effectively when the challenge virus multiplied rapidly. The following sections will deal with the experimental evidence identifying this factor as neutralizing antibody believed to be produced locally in response to the challenge inoculum.

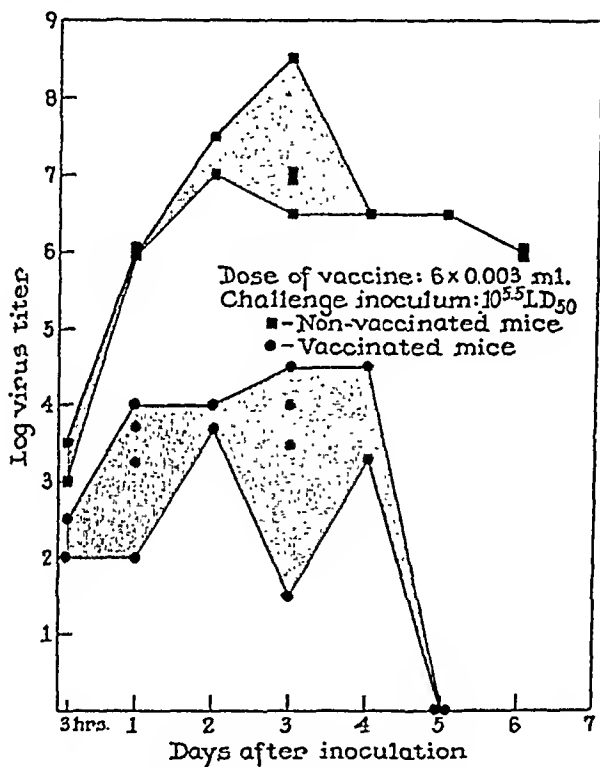


FIG. 2. Multiplication of W.E.E. virus, "slow" Kelser strain, in brains of normal and of vaccinated mice.

Changes in the Serum/Brain Antibody Ratio in Vaccinated Mice after Intracerebral Challenge Inoculation

In continuation of the first experiment described above, the sera and brains obtained from mice at various intervals after challenge inoculation with the "slow" Kelser strain were tested for neutralizing antibody. The outcome of these tests is presented in Table III and Fig. 3.

A pool of 9 sera collected from vaccinated but unchallenged mice at the time of challenge had a neutralizing titer of 1.8 log (1:64). As expected, with a serum titer of such low order,

no significant amounts of antibody could be measured in the corresponding brains even though they were not perfused. Only 3 of them, when tested individually, neutralized in the lowest dilution tested (1:64). Beginning on the 5th day after challenge, the brain titer increased out of proportion to the serum titer with a resulting marked shift in the serum/brain ratio. The brain titer was maintained on a high level from the 7th to at least the 114th day, the ratio decreasing from the "normal" value of about 200/1 to 16/1 on the 6th day to less than 10/1 from the 13th day on. It is particularly noteworthy that the neutralizing titer of brain tissue was relatively high on the 5th day in view of the fact that brains harvested on the 4th day had contained considerable amounts of virus (see Fig. 2).

TABLE II

Multiplication of W.E.E. Virus, "Slow" Strain, in Brains of Immune and of Normal Mice

Dose of vaccine	Time sacrificed after intracerebral inoculation	Lot	No. of brains	Log LD ₅₀ recovered		Mean increase 3 to 48 hrs.
				Single lots	Mean	
0.015 ml.	hrs.					
	3	A	3	3.16	3.18	1.80
		B	3	3.25		
		C	3	3.12		
	48	D	3	5.16	4.98	
		E	3	4.62		
F		3	5.08			
None	3	G	3	3.61	3.41	2.91
		H	3	3.25		
		I	3	3.36		
	48	J	3	6.50	6.32	
		K	4	6.14		

Challenge inoculum, $10^{5.5}$ LD₅₀.

Thus, in animals with a relatively low degree of initial immunity (as indicated by a serum titer of 1:64 just before challenge with virus) the multiplication of a large inoculum of the "slow" strain was arrested, and neutralizing antibody appeared in the brain in excess before the virus had spread to an extent incompatible with the animal's recovery. A causal relationship between the accumulation of antibody in the brain and survival of the animal is suggested by the fact that other animals in the same experiment failed to resist a comparable dose of the "fast" variant which attained lethal concentrations well before the 5th day (see Fig. 1).

As a corollary, in an attempt to show whether or not the neutralizing substance found in brain tissue was antibody, some of the brain suspensions and

TABLE III

Titers of Neutralizing Antibody in Sera and Brains of Vaccinated Mice before and after Intracerebral Challenge Inoculation

Time after challenge inoculation	Mouse No.	Log ND ₅₀				Mean ratio $\frac{\text{Serum}^*}{\text{Brain}}$			
		Serum		Brain		Log	Antilog		
		Single or repeated tests	Mean	Single or repeated tests	Mean				
days									
5	B 15	—	—	1.42	1.42	—	—		
	B 16	—	—	1.42					
6	B 17	2.60, 2.55†	2.58	0.92	<1.36	>1.22	$\frac{>16.6}{1}$		
	B 18			<1.17					
	B 19			1.87					
	B 20			1.49					
7	B 21	—	—	1.72, 1.65	1.69	—	—		
10	B 22	—	—	2.82	2.52	—	—		
	B 23	—	—	2.22					
13	B 24	3.10, 2.60	2.85	2.17, 1.77	1.89	0.96	$\frac{9.1}{1}$		
	B 25			1.82					
24	B 26	3.02, 2.30	2.66	1.82	1.59	0.89	$\frac{7.8}{1}$		
	B 27			2.07					
55	B 28	2.90	2.80	1.42	2.02	0.79	$\frac{6.2}{1}$		
	B 29	2.80		2.17					
	B 30	2.80		2.42					
114	B 31	2.55	2.63	1.80	1.98	0.65	$\frac{4.5}{1}$		
	B 32								
	B 33	2.70		2.15					
	B 34								
	B 35								

Total dosage of vaccine, 0.018 ml. Challenge dose, $10^{5.5}$ LD₅₀ of "slow" variant.

* A pool of 9 sera obtained before challenge inoculation gave a mean ND₅₀ of 1.8 (1:64). The corresponding brains were not perfused, and only 3 of the 9 showed some evidence of neutralization in the lowest dilution tested (1:64). The prechallenge ratio therefore could not be determined and is assumed to be of the usual order; i.e., about 200/1.

† Braces indicate that pools were tested.

For further explanation see Table I.

sera were tested for complement-fixing antibody. The results are shown in Table IV. Complement-fixing antibody was first demonstrated in the brains of mice sacrificed on the 10th day after intracerebral inoculation. On the 13th

day both serum and brain titers were at maximum, and the ratio was 8/1. Thereafter, both titers fell, but the ratio remained at 8/1 on the 24th day and at 4/1 on the 55th day.

Assuming that a "physiological" serum/brain ratio of the order of 200/1 applies to complement-fixing antibody as it does to other types of antibody, the shift in neutralizing titers was paralleled by a similar shift in complement-fixing titers.

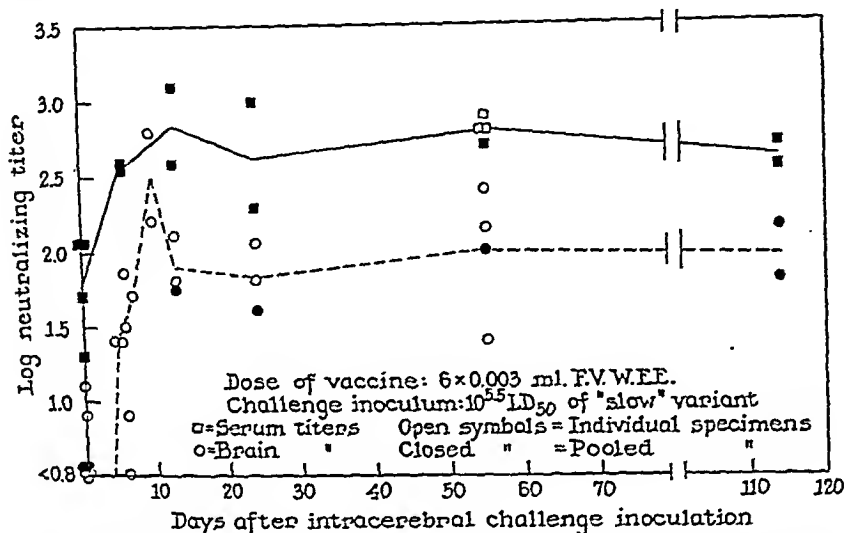


FIG. 3. Titers of neutralizing antibody in sera and brains of vaccinated mice before and after intracerebral challenge inoculation.

Further Evidence that the Neutralizing Substance in Brain Tissue Is Antibody

(a) *The Specificity of the Antibody in Brain Tissue.*—Equal parts of Eastern and Western E.E. vaccines were mixed, and 36 mice were given intraperitoneally a total of 0.15 ml. of the mixture. Two weeks after the first dose of vaccine, 6 mice were sacrificed, 15 received intracerebrally $10^{4.5}$ LD₅₀ of E.E.E. stock virus and the remaining 15 by the same route $10^{5.2}$ LD₅₀ of the "fast" R.I. strain of W.E.E. virus. At 2 weeks after challenge inoculation, there were 8 survivors in the E.E.E. group, but only 3 in the W.E.E. group. All these were bled from the heart, perfused, and their brains were harvested. Sera and brains were pooled in lots as indicated in Tables V and VI and titrated for neutralizing antibody against W.E.E. virus and for complement-fixing antibody against both the Eastern and Western types.

The result of the neutralization test is summarized in Table V. The outstanding finding is that after challenge with W.E.E. virus, the homologous titer of brain tissue rose to at least the same level as that of the serum, while after challenge with E.E.E. virus, the Western antibody remained unchanged in both serum and brain tissue. This finding, as will be discussed later on,

confirms the belief that the accumulation of antibody in the brain is the result of local production.

TABLE IV

The Rise and Fall of Complement-Fixing Antibody in Serum and Brain Tissue of Immune Mice before and after Intracerebral Challenge with $10^{5.5}$ LD₅₀ of the "Slow" Strain of W.E.E. Virus

Interval after intracerebral challenge	No. of mice	Test material	Antigen											
			W.E.E.								E.E.E.			
			Dilution of test material 1:											
			4	8	16	32	64	128	256	512	4	8	16	32
0 (before challenge)	9	Serum	—	3	±	0	—	—	—	—	—	0	0	0
		Brain	0	0	—	—	—	—	—	—	±	0	—	—
6	4	Serum	—	4	4	4	4	3	0	—	—	0	0	0
		Brain	—	0	0	0	0	—	—	—	—	0	0	0
7	1	Brain	—	0*	0	0	0	0	0	—	—	0*	0	0
10	2	Brain	—	3*	1	0	0	0	0	—	—	0*	0	0
13	2	Serum	—	4	4	4	4	4	3-4†	0	—	0	0	0
		Brain	—	4*	4	3-4	0-2	0	0	0	—	0*	0	0
24	2	Serum	—	4	4	4	3-4	0	0	0	—	0	0	0
		Brain	—	2-3	±	0	0	0	0	—	—	0	0	0
55	3	Serum	—	—	4	1	0	0	0	—	—	—	0	0
		Brain	3	1	0	±	0	—	—	—	—	0	0	—
114	5	Serum	—	—	0	0	0	0	0	—	—	—	0	0
		Brain	0	0	0	0	0	0	—	—	—	0	0	—

The results are presented as, 4 = complete fixation; 0 = complete hemolysis with intermediate degrees of fixation expressed as 3, 2, and 1. The highest dilution giving a reading of at least 2 is considered as end-point (bold-faced type).

* Indicates that the lowest dilution tested was 1:10 instead of 1:8.

† Two figures indicate results of duplicate tests.

In Table VI, the outcome of the complement fixation test with the same materials is shown. Here again, the serum/brain ratio for antibody reacting with the virus used for the intracerebral test was reduced to 2/1 to 8/1. In these doubly immunized mice, the antibody response to challenge was just as specific in the brain as in the serum.

(b) *The Percentage Law.*—Table VII illustrates one of several tests in which constant amounts of serum or brain extract of vaccinated and challenged mice

were mixed with graded amounts of virus and in which the resulting mixtures after 2 hours' incubation at 37°C. were diluted serially so that the highest dilution contained 10^{-8} virus. It may be seen that in both cases the percentage of virus neutralized was independent of the amount of virus originally added to the test material. In other words, the "percentage law" as described by Mer-

TABLE V

W.E.E. Neutralizing Antibody in Serum and Brain Tissue of Mice Immunized against W.E.E. and E.E.E. Viruses before and 2 Weeks after Intracerebral Challenge Inoculation of Either Virus

Specificity of the Local Response

Challenge virus	Lot	No. of mice	Test material	Log ND ₅₀	Serum/Brain ratio log
None	A	3	Serum Brain	2.15 <0.70	>1.45
	B	3	Serum Brain	1.80 1.02	0.78
W.E.E.	C	3	Serum Brain	2.80 >2.78	<0.02
E.E.E.	D	4	Serum Brain	1.80 <0.65	>1.15
	E	3	Serum Brain	2.21 <0.90	>1.31
	F	1	Serum Brain	2.07 <1.27	>0.80

The lowest dilution of brain tissue tested was $10^{-0.9}$ for lots A to E, and $10^{-1.4}$ for lot F. In some instances, there was a suggestion of neutralization by these dilutions. The estimated ND₅₀ titers are unduly high because they are based on the assumption that with lower dilutions all mice would have survived.

For explanation of symbols see Table I.

rill (10) for Eastern E.E. virus and its antibody and as observed for other antigen-antibody mixtures (11) applied equally to the neutralizing substance in the brain and that in the serum.

The Effect of Varying Degrees of Immunization on the Shift in Serum Antibody/Brain Antibody Ratio after Intracerebral Challenge

Mice which had been immunized with graded amounts of W.E.E. vaccine and had survived after intracerebral challenge inoculation of varying amounts of the "slow" Kelser

TABLE VI
Complement-Fixing Antibody in Serum and Brain Tissue of Mice Immunized against W.E.E. and E.E.E. Viruses before and 2 Weeks after Intracerebral Challenge Inoculation of Either Virus
Specificity of the Local Response

				Antigen																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																										
Challenge inoculum	Lot	No. of mice	Test material	W.E.E.								E.E.E.																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																		
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None	A	3	Serum Brain	4	3	1	0	—	—	—	—	—	—	—	—	—	—	—	—	—	4	4	4	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

For explanation of figures see Table IV.

* The single mouse of lot C was of special interest because it was sacrificed after a course of encephalitis with gradually improving paresis of the extremities lasting 10 days.

strain, were sacrificed at various intervals. These mice were survivors from the experiment described in Fig. 6 of the preceding paper (4). The sera and perfused brains were each pooled according to the amount of vaccine received. Each pool contained specimens from mice challenged with 10^5 , 10^3 , and 10^1 LD₅₀ of virus.

The results of a neutralization test with these pools are summarized in Table VIII. It is shown that the relative concentration of antibody in the brain increased with decreasing dosage of vaccine used for immunization. As a result, while all the serum titers were of the same order of magnitude, there was a

TABLE VII

The "Percentage Law" in the Neutralization of W.E.E. Virus by Immune Mouse Serum and Brain Tissue

Test material	Original mixture: Final dilution of		Survival rate in mice inoculated with						
	Serum or brain	Virus	Original mixture	Dilution of mixture to final virus concentration equivalent to:					
				10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Immune serum	$10^{-1.3}$	10^{-3}	0/4	0/4	0/4	0/3	1/3	4/4	—
		10^{-4}	0/4	—	0/4	0/4	2/4	3/4	—
		10^{-5}	0/4	—	—	0/4	2/4	3/4	—
		10^{-6}	0/4	—	—	—	0/4	4/4	—
		10^{-7}	2/4	—	—	—	—	4/4	—
		10^{-8}	3/4	—	—	—	—	—	—
Immune brain	$10^{-0.3}$	10^{-4}	0/4	—	0/4	0/4	0/4	4/4	—
		10^{-5}	0/4	—	—	0/4	1/4	3/4	—
		10^{-6}	0/4	—	—	—	0/4	3/4	—
		10^{-7}	1/4	—	—	—	—	3/4	—
		10^{-8}	2/4	—	—	—	—	—	—
Normal mouse serum	$10^{-1.3}$	10^{-3}	0/4	—	—	—	0/4	0/4	3/4
		10^{-7}	0/4	—	—	—	—	0/4	2/4
		10^{-8}	0/4	—	—	—	—	—	—
		10^{-9}	3/4	—	—	—	—	—	—
		10^{-10}	3/4	—	—	—	—	—	—

tenfold difference between the serum/brain ratios in mice vaccinated with 1.8 ml. of vaccine (ratio 24.6/1) and in those vaccinated with 0.0018 or 0.0057 ml. (ratio 2.3/1 or 2.8/1), with intermediate values for those vaccinated with 0.018 or 0.057 ml. A single complement fixation test was carried out with pooled sera and brains from the latter 4 groups. As shown in Table VIII, the ratio was 4/1 which is again in good agreement with that for the neutralizing titers. It is noteworthy that the time interval which elapsed after challenge inoculation did not influence the consistency of the pattern. This is in agreement with the experiment described earlier in which the ratio remained more or less constant from about the 13th to the 14th day after inoculation.

One may assume that in animals immunized with large amounts of vaccine a considerable proportion of the challenge inoculum would be neutralized and thereby rendered incapable of multiplication and also less effective as antigen. Conversely, after vaccination with small doses, the primary antibody response may be minimal but the animal would be sufficiently sensitized to respond to the challenge virus with a large and rapid local output of antibody. By the same token, in highly immunized animals, survival may be largely due to the suppressive effect of antibody initially present which would be effective against either "fast" or "slow" variants of the virus (see Fig. 6 in reference 4). In less

TABLE VIII

Effect of Degree of Prechallenge Immunity upon Local Antibody Response to Intracerebral Challenge Inoculation

Total dosage of vaccine	Sacrificed after challenge	No. of mice in pool	Material tested for neutralizing antibody	Log ND ₅₀	Ratio Serum Brain		Titer of complement-fixing antibody
					Log	Antilog	
ml.	days						
1.8	15	10	Serum Brain	2.80 1.41	1.39	$\frac{24.6}{1}$	n.t.
0.057	22	8	Serum Brain	2.91 1.98	0.93	$\frac{8.5}{1}$	Pooled sera 1:32 Pooled brains 1:8 Ratio $\frac{1}{4}$
0.018	33	12	Serum Brain	2.38 1.55	0.83	$\frac{6.7}{1}$	
0.0057	26	9	Serum Brain	2.38 1.93	0.45	$\frac{2.8}{1}$	
0.0018	127	10	Serum Brain	2.41 2.05	0.36	$\frac{2.3}{1}$	

For explanation of figures see Table I.

n.t., not tested.

thoroughly immunized animals, survival would then depend more and more predominantly on their potential ability to respond to the challenge inoculum with local antibody production, and this in turn would be effective only if the rate of viral multiplication were not too rapid. This complex mechanism in which viral growth competes with local antibody production probably accounts for individual variations often seen among animals vaccinated and challenged in the same manner.

The "Paradoxical" Response of Lightly Immunized Mice to Small Intracerebral Challenge Doses

Another observation in support of the concept just discussed is that of a "paradoxical" response of lightly immunized mice to large or small intracerebral

challenge doses of active virus. Table IX and Fig. 4 illustrate two examples of this. In both experiments, graded doses of the "slow" Kelser strain were injected intracerebrally into mice immunized with very small amounts of vaccine. Table IX shows that mice vaccinated with 0.0057 or 0.0018 ml. resisted 10^3 or 10^3 LD₅₀ but that 4 of 8 succumbed after inoculation of 10^1 LD₅₀. Similarly, Fig. 4 shows the response of mice immunized with 0.0006 ml. of vaccine. While 10 of 24 mice survived after inoculation of 10^3 to 10^5 LD₅₀ of virus, there was no sign of protection among mice challenged with smaller amounts (to the right of the broken line in Fig. 4) when compared with non-vaccinated controls.

It is reasonable to assume that the degree of the local immune response should depend on the amount of antigen injected. While in mice with a higher degree of immunity small doses of challenge virus may be expected to be effective

TABLE IX
"Paradoxical" Response to Challenge Doses of W.E.E. Virus, Slow Strain, in Mice with Low Grade Immunity

Total dose of vaccine ml.	Challenge dose LD ₅₀ of W.E.E.-Kelser virus		
	$10^{1.2}$	$10^{2.2}$	$10^{3.2}$
1.8	1/4*	0/4	0/4
0.18	0/4	0/4	0/4
0.057	0/4	0/4	0/4
0.018	0/4	0/4	0/4
0.0057	0/4	0/4	2/4
0.0018	0/3	0/4	2/4

* Numerator, number of deaths, denominator, number of mice inoculated.

tively blocked by the antibody initially present in the brain tissue, in those with a very low degree of immunity this amount of antibody must be infinitesimal. In the preceding paper (4) the differences in viral growth rate after inoculation of various amounts of W.E.E. virus were described. It was shown that with large seed inocula the rates tended to converge while, in the case of the R.I. strain, with decreasing inocula there was a tendency for the rates to parallel each other. It was assumed that with increasing amounts seeded, a correspondingly larger proportion was in excess of that utilized immediately to initiate infection. This excess could then, in the sensitized animal, act as free antigen. A very small inoculum, on the other hand, if taken up quantitatively by susceptible cells, would be ineffective as antigen, with the result that viral multiplication could proceed unchecked.

How delicate this balance between virus increase and immune response may be is indicated by the consistently high incidence of transitory encephalitic signs among mice with borderline degrees of immunity. This has been graphi-

cally illustrated by shading individual squares in Fig. 4. The mice so symbolized all had definite convulsive seizures and were expected to die. Instead, they recovered completely after a few days or continued to live, sometimes for months, with gradually improving partial paralysis of the extremities.

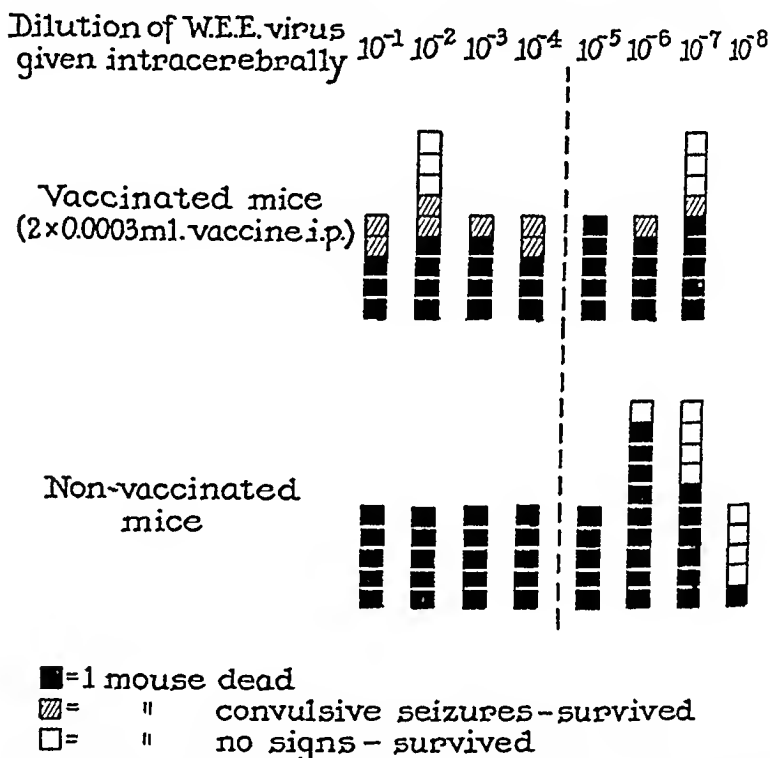


FIG. 4. "Paradoxical" response to graded challenge doses of W.E.E. virus in mice with low grade immunity.

RECAPITULATION AND DISCUSSION

While the virus-inactivating and protective action of neutralizing antibody is readily evident both *in vitro* and in passively immunized animals, its rôle in actively immunized hosts has been a subject of doubt and speculation. This has been due to the inability, especially in the case of neurotropic virus infections, to consistently correlate varying titers of neutralizing antibody acquired as a result of vaccination with corresponding variations in the degree of resistance to infection. This has been especially true for those studies in which the degree of resistance was measured by intracerebral challenge inoculation of virus. Thus, conflicting opinions have been expressed concerning the possible significance of neutralizing antibody in relation to cerebral resistance to the

cases of poliomyelitis (12), equine encephalomyelitis (1, 13), St. Louis encephalitis (13), and rabies (14). Morgan, Schlesinger, and Olitsky (2) showed that vaccinated rabbits were able to resist large intracerebral challenge doses of E.E. virus provided their serum contained neutralizing antibody of high titer. In such animals demonstrable amounts of antibody penetrated into the central nervous system, the serum/brain tissue/spinal fluid ratio being of the order of 300/3/1. It was thought that adequate amounts of antibody available to the exposed tissue at the time of challenge were necessary to insure resistance. Subsequent finding of a shift in this ratio through an increase in the neutralizing titer of brain tissue after challenge inoculation (3) first suggested that local production of antibody might be stimulated by the challenge inoculum itself.

The work reported in this and the preceding paper (4) has revealed a relationship between local antibody accumulation and survival of immune animals. It has led to the following conclusions regarding the mechanism of cerebral immunity to W.E.E. virus:—

(a) The degree of resistance of vaccinated mice to intracerebral challenge inoculation is in part determined by the rate of multiplication of the challenge virus.

(b) In mice immunized with relatively large doses of vaccine, the difference between fast and slow variants of W.E.E. virus is masked, because the high initial concentration of antibody in their serum and central nervous system can effectively block or retard the spread of the virus regardless of its rate of multiplication.

(c) In animals with lower degrees of immunity, the amount of antibody initially present may be inadequate to retard significantly the rate of growth of a "fast" variant of the virus. Because of the rapid rate at which it multiplies there may be no chance for local antibody production to be effective in preventing fatal infection. The "slow" variant, on the other hand, not only affords the immediately available antibody a better chance to react with it, but it also gives time for sufficient additional antibody to be produced locally. Hence even though the virus may multiply and persist in the brain at a relatively high level for several days the animal survives.

(d) As a result of this successful competition of local antibody production with viral multiplication, the serum antibody/brain antibody ratio is considerably reduced.

(e) The degree of this shift in serum/brain ratio varies inversely with the initial degree of immunity: the more antibody available initially the greater the proportion of the inoculum which is rendered inert as antigen.

(f) The intensity of the local immune response is also proportional to the amount of antigen injected as challenge. In animals with extremely low grade immunity, this may lead to a "paradoxical" response to large and small intra-

cerebral challenge doses: while such animals are sufficiently sensitized to give a rapid anamnestic immune response to excessive amounts of virus, small amounts are not sufficiently antigenic and are therefore more apt to cause fatal infection.

The latter finding, in particular, suggests that the antigenic booster effect is exerted chiefly by the virus contained in the inoculum itself. For, as has already been pointed out, the growth rate studies (4) lead one to believe that with increasing amounts of virus inoculated, correspondingly larger proportions of the inoculum exist in excess of the amount utilized to initiate infection. Presumably this excess virus does not attack susceptible cells and is free to stimulate antibody formation. Similarly, after inoculation of small infectious doses, some of the virus liberated by infected cells after multiplication would be free to act as antigen but probably too late to affect the outcome; it would be ineffective in arresting the course of the infection, especially if, as is probable, the virus progressed along the neuronal pathway (15), for in that case virus may already have reached distant neurons at the time when antibody becomes available. It has been shown by Morgan (12) that non-fatal infection of the brain and cord of the monkey with poliomyelitis virus leaves in the wake of its neuronal pathway a trail of high neutralizing titers.

The concept of the mechanism of cerebral immunity just outlined may appear complex, but it is in accord with generally accepted principles of immunology (16). Local accumulation of specific antibody at the site where antigen is deposited has been described by Walsh and Cannon (17) and DeGara and Angevine (18) for bacterial agglutinins and by Oerskov and Andersen (19) and Hartley (20) for antivaccinal antibody. Similarly, local concentration of neutralizing substances, presumably antibody, in the central nervous system after infection with viruses has been reported by Fox (7) for yellow fever in mice and by Morgan (12) for poliomyelitis in monkeys. The present study leaves no doubt that the neutralizing substance present in high concentration in the brain tissue of mice immune to and challenged with W.E.E. virus is antibody: (a) it reacts specifically with homologous antigen, (b) it follows the "percentage law" on dilution of underneutralized mixtures, (c) its concentration in relation to that in the serum is paralleled by that of complement-fixing antibody.

The finding of complement-fixing antibody of high titer in blood-free brain tissue is a novel observation in the field of virus diseases. It is reminiscent of the presence of complement-fixing antibody in the cerebrospinal fluid of neurosyphilitic patients. Kabat, Moore, and Landow, on the basis of electrophoretic comparison of serum and cerebrospinal fluid proteins, came to the conclusion that this antibody is produced within the central nervous system (21). This interpretation has been favored also by Morgan (12) and Fox (7) for the systems studied by them, and evidence presented in this paper lends significant support to it: that is the observation that in mice vaccinated with a mixture of

inactive Western and Eastern E.E. viruses and challenged intracerebrally with active virus of either type, the relative concentration of the homologous, but not of the heterologous antibody in brain tissue rises. Such specificity could not be expected in doubly immunized animals if the local accumulation of antibody were assumed to be due to increased permeability of capillary walls or to some non-specific disturbance in the protein balance between blood and brain tissue.

An interesting problem for further investigation is the reason for the long persistence of local antibody in high concentration. In the present report, 127 days was the longest interval after intracerebral inoculation of W.E.E. virus at which the ratio serum titer/brain titer was examined, and in that particular experiment (see Table VIII) it had remained at 2.3/1. Similarly, Morgan (12) found the neutralizing titer against the Lansing strain very high in monkey cord as long as 5 months after onset of paralysis. Earlier observations on the markedly delayed onset of encephalitis in W.E.E.-infected guinea pigs treated with large doses of hyperimmune serum (22) suggested that in passively immune animals the virus sometimes persisted in the central nervous system masked by antibody which blocked the development of active immunity. In contrast, persistence of antigen in actively immune animals may be responsible for continued presence of local antibody in high titer.

The "paradoxical" type of response of mice with low degree of immunity to various amounts of active virus, *i.e.* their failure to survive minimal challenge doses although they can resist large ones, is not without parallel. Casals (23) and Habel and Wright (24) have noted the same phenomenon in mice vaccinated and challenged with rabies virus, and the explanation given here for the findings with W.E.E. virus may apply equally for rabies.

In general, there is no reason to believe that the principles found to govern the mechanism of cerebral immunity to W.E.E. virus should not be applicable to the wider field of other virus infections of the central nervous system and of other organ systems. The findings reported in the preceding paper (4) and in this one reaffirm that the actively immunized animal differs from the normal one not in principle but only in its greater ability to respond to an antigenic stimulus. The fate of the immune host after challenge—even that of the mouse inoculated intracerebrally with W.E.E. virus—is not either death or intact survival. There may be any intermediate degree of involvement. What happens is determined by a delicate quantitative balance with relation to (a) degree of initial immunity or sensitization, (b) amount of the challenge virus, (c) rate of its multiplication, (d) ability of the host to respond with local antibody production.

SUMMARY

The fate of W.E.E. virus has been followed in the brains of mice vaccinated to such an extent that they failed to resist a large intracerebral challenge dose

of a viral variant with a rapid rate of multiplication but were fully protected against a similar amount of a "slow" strain.

The growth rate of the "fast" variant in vaccinated animals paralleled that in non-vaccinated ones at a slightly lower level. The "slow" strain also multiplied, but its rate of growth was depressed. Nevertheless, it persisted for 4 days at a level 100-fold higher than its initial titer.

After the 4th day the virus was no longer demonstrable and was replaced by neutralizing antibody which rose so high that the serum antibody/brain antibody ratio was reduced from a "physiological" value of about 200/1 to less than 10/1. Antibody persisted in brain tissue in high titer until at least 127 days after challenge inoculation.

The shift in the serum/brain ratio of neutralizing antibody was paralleled by a similar shift in the ratio of complement-fixing antibody.

The neutralizing antibody in brain tissue, like that in serum, followed the "percentage law" on dilution of underneutralized mixtures.

In mice immunized with small doses of vaccine, the intracerebral challenge inoculum induced a significantly greater local immune response than in those immunized to a higher degree.

Mice with very low grade immunity were found more resistant to large amounts of virus than to small amounts. This "paradoxical" response to challenge was explained as due to the antigenic booster effect exerted by amounts of virus in excess of that utilized to initiate infection which were present in large inocula but absent in small doses.

The broader relation of these findings to the problem of antiviral immunity has been discussed.

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NUTRITION OF THE HOST AND NATURAL RESISTANCE TO INFECTION

IV. THE CAPABILITY OF THE DOUBLE STRAIN INOCULATION TEST TO REVEAL GENETICALLY DETERMINED DIFFERENCES IN NATURAL RESISTANCE TO INFECTION

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Among students of infectious disease the term "natural resistance" has come to include those attributes of a host, preexistent to the actual event of infection, which tend to lessen the consequences of that infection. It is an obvious thesis that an analysis of these mitigating host attributes might lead to possibilities of their enhancement to the happy end (from the host's view) that the effects of infection might thus be reduced from the serious or fatal to the trivial or inapparent. From a theoretical viewpoint these host attributes may be due to (a) genetic or (b) environmental causes. The diet a host consumes is one such environmental factor of possible importance and in investigations previously reported (1-3) an analysis has been undertaken of the biological circumstances in which the nutrition of the host is capable of favorably influencing the frequency of survivorship following an initial infection. The disease model employed in these studies was mouse salmonellosis and from the findings it was concluded that host nutritional influences were operative in a genetic framework. This genetic framework was constructed of the various genotypes within the host and pathogen species as they joined in various combinations in the infection experience (see reference 2). In each of these unique collisions of a certain pathogen genotype with a certain host genotype the effect of host nutrition on survivorship frequency had been examined but in this examination a choice was made as to the kind of nutritional difference which was tested. This choice was, in a sense, a limitation on any statements which might be made on the effects of nutrition in general on these events, for the list of nutritive entities which might be manipulated, either singly or in combinations, is almost endless. But in order to begin somewhere, and with the most encouraging prospect, recourse was had to the historical precedent in the development of the science of nutrition. The nutritional difference which was chosen for testing for its ability to bring about differences in survivorship frequency following infection was the nutritional gap existing between a so called "synthetic" diet and a diet of natural foodstuffs.

When the effect of nutrition had been tested in this manner the greatest

increment of increased survivorship on the natural diet was observed when genetically heterogeneous hosts were infected with a pathogen population which in itself was heterogeneous in its genotypic composition with respect to virulence. Further examination of this rôle of pathogen heterogeneity in the nutrition effect led to a change in technique in the experiments designed to permit the concentration and isolation of the factor(s) in the natural diet responsible for the increased frequency of survivorship. This change in technique exploited the concept of the heterogeneous pathogen by employing two different cultures of *Salmonella typhimurium*, one avirulent and the other virulent, and further, by injecting into the host the avirulent culture 24 hours before the virulent one. This procedure has been named the double strain inoculation test, or DSI test. The steps which led to this infection procedure have been described previously (3), but it is worth emphasis that this selection of a 24 hour time interval between the two components of the heterogeneous pathogen population bears only a special relevance for these two particular cultures and this relevance rests solely on the fact that it is at precisely this time interval that the dietary effect is maximal (see reference 3). For other cultures other time intervals, including the zero time interval (see reference 2), might well prove to be maximal in their effect.

Whatever may be the specific details which the DSI test might take in any specific infection the fact remains that its important element of conducting an infection test of "natural resistance" by means of two separate cultures was arrived at by an analysis of the effect of an environmental factor; *i.e.*, diet. The question now arises whether this test can detect differences in "natural resistance" in those instances in which these host attributes are determined not by environment, but by genetics. For there is no evidence, *a priori*, which would assure that differences in "natural resistance" arranged by diet are necessarily the same as differences arranged by genetic composition. Indeed it has been found necessary to devise a special test to detect these diet effects with reliability. What we need to know is whether the DSI test is capable of detecting these genetically arranged differences in survivorship as well as the nutritionally arranged event, or whether it is incapable of so doing and we are thereby reduced to considering the DSI method as dealing fundamentally with something different from the genetically controlled situation. To find an applicability of the DSI method in both the genetic and the nutritional event would have certain advantageous consequences, not the least of which is that it would make possible the beginnings of a unified biochemical theory which would underlie both the nutritional and genetic factors in their separate workings.

It is the substance of this paper that the DSI method is capable of revealing differences in "natural resistance" which are referable to genetic constitution in addition to its established ability to reveal differences in "natural resistance" due to nutrition.

Materials and Methods

Animals.—In the following experiments use has been made of four pure, brother-sister inbred strains of laboratory mice (18th to 29th generation) developed in this laboratory by the late Leslie T. Webster from a common stock of Rockefeller Institute mice and now differing widely in their response to injection. These stocks are maintained free of *Salmonella* infection. The derivation of these strains has been described by Webster (4, 5). Briefly, they are characterized by the four possible combinations of relative resistance and susceptibility to a bacterial disease (mouse typhoid, salmonellosis), and to a virus disease (St. Louis encephalitis); *viz.*, bacteria-resistant, virus-resistant; bacteria-susceptible, virus-resistant; bacteria-resistant, virus-susceptible; and bacteria-susceptible, virus-susceptible. The response of these strains to the two different kinds of infection, under the conditions of this laboratory,

TABLE I

*Survivorship of Inbred Strains of Mice Selected for Resistance or Susceptibility to Infection with S. enteritidis or St. Louis Encephalitis Virus**

Description of strain	Strain designation	Test pathogen	No. of mice tested (in 1942)	Survivorship†
				per cent
Bacteria-resistant, virus-resistant	BRVR	<i>S. enteritidis</i>	449	92
		St. Louis encephalitis	606	76
Bacteria-susceptible, virus-resistant	BSVR	<i>S. enteritidis</i>	86	2
		St. Louis encephalitis	98	88
Bacteria-resistant, virus-susceptible	BRVS	<i>S. enteritidis</i>	287	82
		St. Louis encephalitis	519	2
Bacteria-susceptible, virus-susceptible	BSVS	<i>S. enteritidis</i>	285	2
		St. Louis encephalitis	271	3

* Adapted from Schneider and Webster (1).

† Testing dose: *S. enteritidis*, 5,000,000 by stomach catheter; St. Louis encephalitis, 10^{-2} suspension of infected mouse brain, intranasally.

is epitomized by data presented in Table I. This table was compiled from data obtained during 1942 when the mice were in their 15th to 20th generation of brother-sister inbreeding. This system of inbreeding has been rigorously followed to the present. Although St. Louis encephalitis virus was not used in any of the experiments reported here, the mouse strain designations employed previously have been retained in this paper for the sake of uniformity with previous publications.

Pathogen.—Two cultures of *Salmonella typhimurium* (IV, V, VII: i-1, 2, 3) were used. One culture, TMO-S3, was relatively avirulent for uniformly susceptible mice. The second culture, BA₂SC-1, was highly virulent for mice. These cultures were derived (see reference 3) from cultures originally received from Dr. G. M. Mackenzie of the Mary Imogene Bassett Hospital of Cooperstown, New York. The present avirulent TMO and virulent BA₂ cultures of Mackenzie, although differing widely in virulence, were both smooth and indistinguishable in cultural, serological, immunizing, and toxigenic characters, and did not differ significantly in invasiveness and resistance to phagocytosis (Pike and Mackenzie (6)). All cultures were

perpetuated by preparing nutrient agar stabs with broth cultures of the organisms, incubating for 24 hours at 37.5°C., and then storing in the ice chest at 4°C. Fresh transfers were made at approximately monthly intervals.

Cultures were prepared for use in the infection experiments by seeding meat infusion broth tubes (10 ml.), incubating for 6 hours at 37.5°C., transferring a loopful to fresh broth (10 ml.) and incubating for 18 hours. The optical density of the cultures was then measured in an Evelyn photoelectric densitometer and the density of viable cells estimated (± 10 per cent) with the aid of a chart prepared from calibration data assembled for each of the two stock cultures. The appropriate dilutions were made with sterile saline, so that the desired dose of cells was suspended in a volume of 0.25 ml., which was injected intraperitoneally into each mouse. As a further check on dosage dilution plates were poured. In all instances these indicated that the administered dosage was as calculated from the densitometer data.

Environment.—All the experiments have been performed in the two air-conditioned rooms previously described (1). These rooms provide a constant temperature of $80 \pm 0.5^\circ\text{F}$. and a constant relative humidity of 50 ± 3 per cent. The artificial lighting (fluorescent) is 12 hours per day, 6 a.m. to 6 p.m. The details of animal caging were the same as previously described (1).

Diet.—All the animals in these experiments were fed our standard laboratory stock diet plus distilled water *ad libitum*. This diet is Irwin's modification (4) of the Steenbock stock diet. It consists of the following items, listed in parts by weight: yellow corn meal, 64; linseed oil meal, 16; crude casein, 5; ground alfalfa meal, 2; powdered dried whole milk, 5; wheat germ, 10; dried yeast, 2; sodium chloride, 0.5; calcium carbonate, 0.5; cod liver oil, 2.

Under the environmental conditions already described the mortality risk of uninfected mice on this diet was nil from weaning age until the time when the experiments here to be described had been completed.

The Double Strain Inoculation Test Applied to BRVS and BSVS Mice

The opportunity to test the double strain inoculation method as a detector of differences in natural resistance due to genetic composition of the host was made possible by the presence in our laboratory of four different inbred strains of mice, the descendants of Webster's selected stocks (4, 5), which differed widely in their response to *Salmonella* infection, as measured by survivorship frequency. As Table I shows, two strains were "resistant" and two "susceptible." These divergent responses to infection were arranged by the geneticist's operation of inbreeding and selection, the guide to selection, be it noted, having been the response to an infection test performed with a single culture of *Salmonella*. The differences between these strains have been established as being innately, or genetically, determined (5). Indeed, the very fact that such stocks exist has been regarded by Zinsser, Enders, and Fothergill (7) as "the most trustworthy evidence which we possess at present for regarding it (inherited resistance) as a real phenomenon."

Before applying the double strain inoculation method to these inbred strains of mice it might be well to recapitulate the basic facts of the double strain phenomenon which underlie the experimental details. These relationships are well brought out by an examination of the effect of various time intervals between the initial admission of the avirulent pathogen population into the

mouse and the final admission of the virulent population. In such circumstances it has been found (3) that the frequency of survivorship increases rapidly with increase of the time interval between the avirulent and virulent doses. It was found further (3) that this rising curve of survivorship frequency was determined by the dietary status of suitable genetically heterogeneous mice, such curves rising more steeply if the mice were on a natural diet than when the mice were on a synthetic diet. The important point is that the DSI test in this manner revealed a divergence in the disease response of the mice on the two different diets. It followed, of course, that the mice which manifested the more steeply rising curve of survivorship frequency were designated as

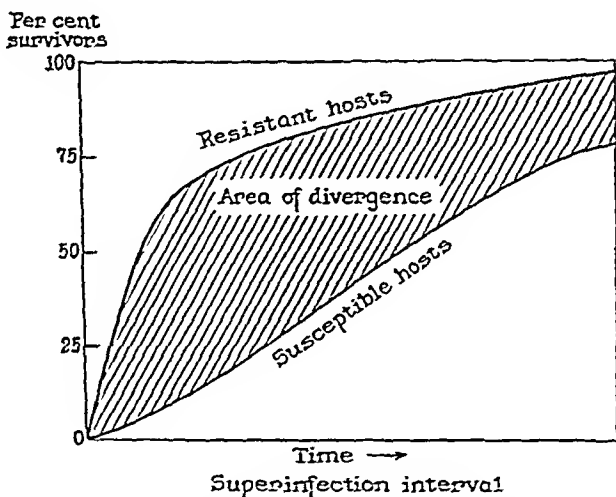


FIG. 1. Schematic diagram of the divergence, with time, of survivorship frequency of resistant and susceptible hosts as revealed by the double strain inoculation test.

"more resistant." The area described between these two rising curves of survivorship frequency is thus an effective index of the difference between the effects of the two diets on the mice at test. These considerations can be represented diagrammatically as in Fig. 1. Although in previous experiments (3) the area of divergence was due to the effect of diet difference (same mice on two different diets), this same divergence should emerge when the double strain inoculation test is performed with mice differing innately in natural resistance (two different kinds of mice on the same diet).

140 weanling BRVS mice (70♂, 70♀), and 140 BSVS mice (70♂, 70♀), were assembled in Room 1, as they became available, over a period of a month. During this period of assembly and throughout the course of the experiment the mice were fed the modified Steenbock stock diet and distilled water *ad libitum*. Four weeks after the last mice had been admitted to their respective assembly, the mice were transferred to Room 2 and placed in individual

cages. The mice were 2 to 3 months old at this point and the average body weight was as follows: BRVS, males 25.9 gm., females 22.1 gm.; BSVS, males 25.2 gm., females 21.4 gm.

Upon transfer to Room 2 the mice were divided by sex, age, and strain into 7 groups of 40 each, composed of subgroups of 20 BRVS and 20 BSVS mice. After 5 days of acclimatization to the new caging, the mice were infected with *S. typhimurium* in the following manner.

One group, the avirulent control, was injected intraperitoneally with 0.25 ml. of a sterile saline suspension of 10^3 viable cells of TMO-S3. A second group, the virulent control, was similarly injected with 10^5 BA₂SC-1. The third group was injected with 0.25 ml. of sterile

TABLE II

Survivorship of BRVS and BSVS Mice after Intraperitoneal Injection of Avirulent TMO-S3, Followed after Various Time Intervals by Virulent BA₂SC-1

Superinfection interval	Dose TMO-S3	Dose BA ₂ SC-1	Mouse strain	S/I*	%S†	Survivorship difference	P
days						per cent	
Controls	10^3		BRVS	18/20	90	0	
			BSVS	18/20	90		
		10^5	BRVS	0/20	0	0	
			BSVS	0/20	0		
0	10^3	10^5	BRVS	0/20	0	0	
			BSVS	0/20	0		
1	10^3	10^5	BRVS	9/20	45	25	>0.1
			BSVS	4/20	20		
2	10^3	10^5	BRVS	15/20	75	50	<0.01
			BSVS	5/20	25		
7	10^3	10^5	BRVS	15/20	75	5	>0.9
			BSVS	14/20	70		
14	10^3	10^5	BRVS	19/20	95	25	>0.05
			BSVS	14/20	70		

* S/I, survivors/infected in test.

† %S, per cent survivors.

saline containing 10^3 TMO-S3 and 10^5 BA₂SC-1. On the same day groups 4 through 7 were injected with 10^3 TMO-S3 and after intervals of 1, 2, 7, and 14 days respectively, groups 4, 5, 6, and 7 were separately superinfected with injections of 10^5 BA₂SC-1.

All mice were observed for 30 days following the last injection of virulent BA₂SC-1 and were then discarded. Deaths were recorded daily. Mice which were alive at the end of the 30 day period were counted as survivors. Results are presented in Table II and Fig. 2.

The determining influence of the genetic constitution of the host for survivorship after double infection with virulent and avirulent *S. typhimurium* is clearly reflected in the data presented in Table II and, graphically, in Fig. 2. Just as in the diet experiments previously reported (3), survivorship is a func-

tion of the time experience of the avirulent *S. typhimurium*, in this instance for both strains of mice; but the resistant strain is characterized by the more rapid rate at which this increased frequency of survivorship is achieved. The designation then of the BRVS strain as a strain naturally resistant (relatively) to *Salmonella* infection is a valid one when tested by the DSI test; or conversely, if the BRVS strain is a satisfactory example of the phenomenon of natural resistance to infection, then the DSI test method is a valid method of identifying stocks of this sort. In order to confirm and extend this conclusion a second test was performed in which a second resistant strain of

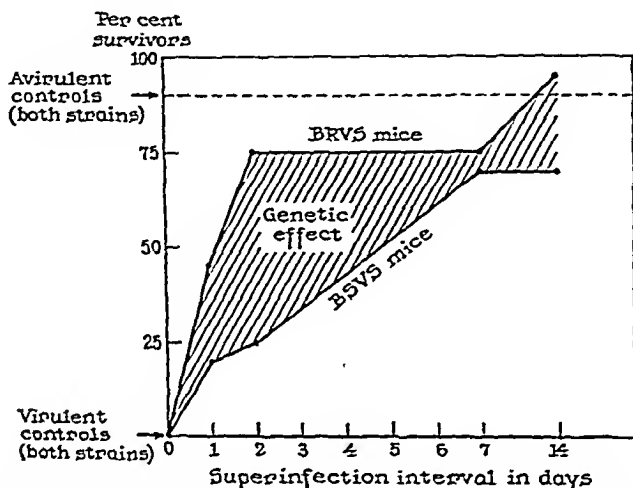


FIG. 2. Survivorship of BRVS and BSVS mice on a modified Steenbock diet after intraperitoneal injection of 10^3 avirulent TMO-S3 followed by 10^5 virulent BA₂SC-1 after various times.

mice, the BRVR strain, was compared with the same susceptible strain, BSVS, used above.

180 weanling BRVR mice (90 ♂, 90 ♀), and 180 weanling BSVS mice (90 ♂, 90 ♀) were assembled in boxes in Room 1, as they became available, over a period of 1½ months. During this period of assembly, and throughout the course of the experiment the mice were fed the modified Steenbock stock diet and distilled water *ad libitum*. Four weeks after the last mice had been admitted to their respective assembly, the mice were transferred to Room 2 and housed in boxes with beddings of wood shavings. At this transfer the mice were divided by sex, age, and strain into 9 groups of 40 each, composed of subgroups of 20 BRVR and 20 BSVS mice. They were housed in boxes of 10 each. After 5 days the mice were infected with *S. typhimurium* in the following manner.

One group, the avirulent control, was injected intraperitoneally with 0.25 ml. of a sterile saline suspension of 10^3 viable cells of TMO-S3. A second group, the virulent control, was similarly injected with 10^5 BA₂SC-1. The third group was injected with 0.25 ml. of sterile saline containing 10^3 TMO-S3 and 10^5 BA₂SC-1. On the same day groups 4 through 9 were

injected with 10^3 TMO-S3 and after intervals of 1, 2, 3, 7, 14, and 21 days respectively, groups 4, 5, 6, 7, 8, and 9 were separately superinfected with injections of 10^5 BA₂SC-1.

TABLE III

Survivorship of BRVR and BSVS Mice after Intraperitoneal Injection of Avirulent TMO-S3, Followed after Various Time Intervals by Virulent BA₂SC-1

Superinfection interval	Dose TMO-S3	Dose BA ₂ SC-1	Mouse strain	S/I*	%S†	Survivorship difference	P
days						per cent	
Controls	10^3		BRVR	14/20	70	0	
			BSVS	14/20	70		
		10^5	BRVR	0/20	0	0	
			BSVS	0/20	0		
0	10^3	10^5	BRVR	0/20	0	0	
			BSVS	0/20	0		
1	10^3	10^5	BRVR	6/20	30	25	<0.1
			BSVS	1/20	5		>0.05
2	10^3	10^5	BRVR	15/20	75	50	<0.01
			BSVS	5/20	25		
3	10^3	10^5	BRVR	15/20	75	35	<0.1
			BSVS	8/20	40		>0.05
7	10^3	10^5	BRVR	15/20	75	-5	>0.7
			BSVS	16/20	80		
14	10^3	10^5	BRVR	12/20	60	-10	>0.5
			BSVS	14/20	70		
21	10^3	10^5	BRVR	16/20	80	-10	>0.5
			BSVS	18/20	90		

* S/I, survivors/infected in test.

† %S, per cent survivors.

All mice were observed for 30 days following the last injection of virulent BA₂SC-1 and were then discarded. Deaths were recorded daily. Mice which were alive at the end of the 30 day period were counted as survivors. Results are presented in Table III and Fig. 3.

It is apparent that the double strain inoculation procedure applied to a second inbred resistant strain of mice and compared with the same inbred susceptible strain has produced results which are similar to those reported above. As Fig. 3 shows, an area of divergence of survivorship frequency was

readily demonstrated and to that extent the claim that the DSI test is a test capable of revealing differences in natural resistance genetically determined is thereby strengthened.

It was next planned to study the relationship between a resistant strain and the second available susceptible strain, the BSVR strain. A difficulty arose, however, when it was found that the susceptibility of this BSVR strain is so great that when injected with 10^3 "avirulent" TMO-S3, all the mice died. Appreciable survivorship frequencies could be obtained only by reducing the dosage of TMO-S3 to 10^1 , but this dose was so small, and the results were so erratic, it was feared that a large doubt had arisen whether, at such small

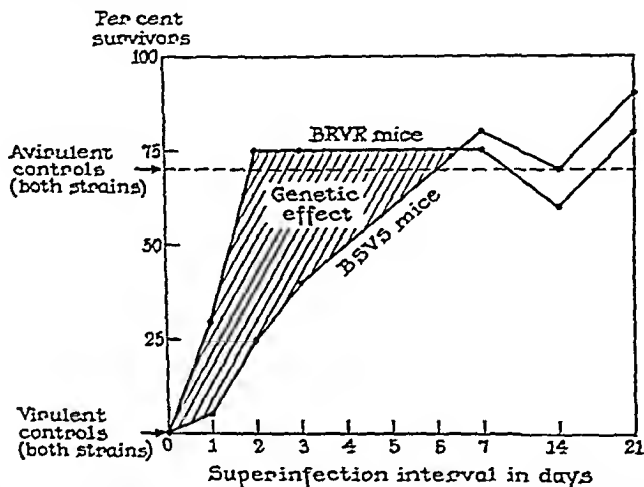


FIG. 3. Survivorship of BRVR and BSVS mice on a modified Steenbock diet after intraperitoneal injection of 10^3 avirulent TMO-S3 followed by 10^5 virulent BA₂SC-1 after various times.

dosages, all the animals were indeed experiencing a controlled contact with the bacterium. The DSI studies with BSVR mice were therefore abandoned. This experience, however, did bring to light a difference between the two susceptible strains, BSVR and BSVS, which had not been known to exist on the basis of the response to the original selection method with single cultures of *S. enteritidis*. (See Table I.)

DISCUSSION

From the data which have been presented above it is evident that those pre-existent host attributes present in mice which mitigate the effect of *Salmonella* infection, and which we designate as "natural resistance," are detectable by the double strain inoculation test whether those attributes are, in certain instances, determined by diet, or whether, in other instances, those attributes are genet-

ically determined. Although these facts may make permissible the use of the double strain inoculation test in studies of natural resistance, is this test a compulsory one? It would not appear to be so, for in Webster's hands (4, 5) a single culture of *Salmonella enteritidis* served satisfactorily for the selection of naturally resistant and susceptible mice. An explanation for this may be that Webster's cultures were not homogeneous, but heterogeneous and hence are not logically considered as single cultures, but as mixtures containing, in all probability, both virulent and avirulent cells. This is constructing an argument after the fact but it is a defensible argument as the following considerations will show.

In all the mouse infection tests which were necessarily performed during the approximately 10 year period that the resistant and susceptible stocks were being inbred and selected (4, 5) the bacterial tests were all performed with the same culture of *S. enteritidis* (*B. enteritidis*) bearing the laboratory designation of MT-1. This laboratory strain of MT-1 was first isolated by Lynch (8) in 1918 from a mouse epizootic in the breeding stock for cancer of the Rockefeller Institute. Twelve years later Webster began his breeding work and used this same culture which had been maintained by transfer at approximately monthly intervals and storage on nutrient agar in the ice chest. It is very unlikely, in the light of modern knowledge of bacterial dissociation, that a laboratory culture of this history could be considered as being homogeneous.

Further evidence that the laboratory culture of MT-1 was not homogeneous, but heterogeneous, was obtained in our laboratory in 1944 (2).

From these considerations it is apparent that a single dose of a mixed culture of pathogen variants can indeed serve as a method for measurement of natural resistance. Whether such a mixed culture serves satisfactorily will depend, among other things, on the relative proportions of the variants. In the zero time mixtures employed in the present experiments for example, the proportion of 10^3 avirulent and 10^5 virulent does not serve as a satisfactory single dose test since all the animals, irrespective of strain, died. This is probably not only a reflection of an imbalance between the two variants, weighted in favor of the virulent form, but also of other fundamental differences between the two variants which are concomitant with the artificial nature of their derivation and culture. The point is that by exploitation of the time curves a satisfactory relationship can be found. Historically, it would appear, there has been a dependence upon the usual heterogeneity of laboratory cultures for the testing of natural resistance differences. But this dependence places its reliance on factors left more or less uncontrolled, such as the relative proportions of the avirulent and virulent variants, to mention one factor. Such uncontrolled oscillations of variant frequencies, due to genetic drifts in the bacterial population, may account for Webster's experience with his inbred strains when he reported (5) "Control measures are frequently inadequate to prevent variation

in results such as the sudden increase of 15 to 20 per cent in mortality percentages in all lines recorded in the present experiments." Only the separate cultivation of avirulent and virulent variants makes possible the direct control of their respective frequencies in a mixture. However, when single cultures of unknown patterns of heterogeneity have been used, such instances can be regarded as constituting a special case of the double strain inoculation test; i.e., the superinfection time interval has been reduced to 0. The utility of the double strain inoculation test is thus based, in part, on its generality and the DSI test is therefore not compulsory, but, for practical reasons, desirable.

SUMMARY

The double strain inoculation (DSI) method of testing for natural resistance to infection has been examined in the instance of mouse salmonellosis. The DSI method has been found capable of detecting differences in natural resistance due to genetic as well as nutritional causes.

A difference in response to *Salmonella* infection was found for the first time between the two "susceptible" inbred mouse strains, BSVR and BSVS. Whereas BSVS mice for the most part survived an intraperitoneal injection of 10^3 "avirulent" *S. typhimurium*, BSVR mice all succumbed.

The relationship of the DSI test to the usual single infection test has been discussed and it is suggested that such single infection tests are special cases of the DSI test, since they involve a heterogeneous bacterial population which can be considered as a mixture of cultures of differing virulence and in which, by a single injection, the usual time interval between the two injections of the DSI method has been reduced to 0.

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DIFFUSE GLOMERULONEPHRITIS PRODUCED IN RABBITS BY MASSIVE INJECTIONS OF BOVINE SERUM GAMMA GLOBULIN*

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PLATES 22 AND 23

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In the present study, interest was directed toward three aspects of experimental acute diffuse glomerulitis. In the first place, an experimental technique was sought which would produce pathological changes morphologically analogous to those of human glomerulonephritis. In the choice of a method the main objective was to employ one which would give a high incidence of well developed lesions. It was also desired to use a technique as uncomplicated as possible, in order that the relation between lesions and treatment might be more easily perceived and assessed. Secondly, it seemed important to examine, in an experiment wherein neither renal substance nor antirenal serum was used, the possible rôle of antikidney antibodies (autoantibodies) in the development of experimental acute diffuse glomerulitis. The third point in this investigation was concerned with the possibility that changes in blood coagulability might play a rôle in the development of diffuse glomerulitis. This report is accordingly divided into three parts, dealing with each of these aspects separately.

1. Production of Experimental Glomerulonephritis

Treatment of experimental animals by a variety of methods has produced renal lesions resembling, to a greater or lesser degree, those of human glomerulonephritis. Nephritis has been produced by injection of various bacteria or their products into suitable animals (1-7). Diffuse glomerular damage has also been evoked experimentally by the injection of non-bacterial foreign protein (8-10). A considerable amount of experimental work has been done using the method of Masugi (11-13), whereby diffuse glomerulitis is produced by injection of specific antirenal antibodies (14-21). The renal lesions produced in this way seem to be the closest morphological equivalent to human glomerulonephritis yet produced.

More recently Hawn and Janeway (22) reported acute diffuse glomerular

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damage in rabbits following intravenous injection of a single massive dose of purified bovine serum gamma globulin (fraction II). In their animals glomerular damage was most marked and acute 1 week after injection. "Healing" lesions were found in animals killed later than this. Unfortunately the illustrations of glomerular lesions in their publication are not such as to constitute convincing evidence of an injury closely resembling human glomerulonephritis.

It was apparent that if this experimental technique could be modified in such a way as to produce more severe renal lesions it would constitute a valuable contribution to the experimental study of glomerulonephritis. The advantage of such a method over others in the experimental study of nephritis lies in the fact that it involves the use of but a single, purified antigen, whereas all other techniques employ complex antigen mixtures; *e.g.*, horse serum (8-10), duck serum (11, 12), bacteria (1-7), bacteria and kidney mixtures (23-26). The use of the single antigen method would reduce considerably the number of factors to be evaluated in understanding the pathogenesis of this type of nephritis.

Method.—Twenty-eight rabbits were used in all. Of these, eighteen were treated with bovine serum gamma globulin,¹ and ten were used as controls. Animals of different strains and both sexes were used. They had an average weight of about 2,500 gm. at the time treatment was instituted. Before receiving any treatment all animals were unilaterally nephrectomized. This was done because of the frequent observation that unilateral nephrectomy increases the susceptibility of the remaining kidney to damage (12, 27). Nephrectomies were done from 3 to 6 weeks before globulin treatment was begun. The animals were given two massive intravenous injections of globulin instead of the single large dose used by Hawn and Janeway. There was a 12 day interval between the two massive injections. Possible fatal anaphylactic reactions were avoided by desensitizing the animals with a small intravenous injection of globulin about 18 hours before they were to receive the second massive dose.

The animals were injected intravenously with 1 gm. per kilo of bovine serum gamma globulin, as a 10 per cent solution in normal saline. Eleven days later they were slowly injected intravenously with the desensitizing dose, consisting of 1.0 cc. of the concentrated solution diluted to 5 or 10 cc. total volume in normal saline. On the following day, a second massive intravenous dose was given, equalling in amount the initial dose.

Blood urea nitrogen estimations were made on eight animals before treatment was begun, and again immediately before the animals were killed. Similar estimations were done on two other animals before killing only. All animals were killed by intravenous air injection 1 week after the second large dose of globulin. Autopsies were performed immediately, and tissues were fixed in Bouin's and Helly's fluids. Histological sections were cut at 3 to 4 μ thickness, and stained with hematoxylin and eosin, hemalum-phloxin-saffranin, Mallory-Haidenthain, Mallory's phosphotungstic acid hematoxylin, and according to McManus' periodic acid routine (28).

Controls.—Ten animals comprised the control group. All were unilaterally nephrectomized.

¹ The supply of bovine serum gamma globulin (fraction II) used in these experiments was kindly supplied to us through the courtesy of the Biochemical Sales Research Department, Armour and Company, Chicago.

Two developed postnephrectomy infections and were killed and autopsied 1 month after operation. Three animals of the control group were allowed to survive nephrectomy for 7 months, in order to control the possibility of spontaneous appearance of renal lesions following this treatment alone. The remaining five animals were treated in exactly the same manner as those of the experimental group, with the exception that instead of injections with bovine serum gamma globulin, they received equivalent amounts of normal saline.

All control animals were killed and autopsied by the same technique as was used in the globulin-treated group, and histological preparations from these animals formed the basis for estimation of the degrees and types of damage found in the treated group.

Results.—Fourteen (77.8 per cent) of the eighteen globulin-treated animals showed gross and microscopic evidence of diffuse glomerulitis in the remaining kidney. It was noted in the gross that the organ was enlarged. However, this increase in size was not significantly greater in the globulin-treated animals than in the nephrectomized controls. Kidneys from the treated animals were yellower and paler than normal. On section most kidneys showed a distinct cortical pallor. In those that proved on microscopic examination to have severe lesions, it was noted that the fine red peppering of the cortex corresponding to the glomeruli, which is normally visible in the gross, was no longer distinguishable. Two of these kidneys showed fine pitting of their external surfaces.

Among the fourteen animals with glomerulitis, the histological changes varied from slight to very severe. Three showed very severe diffuse glomerulitis (++++), two were moderately severe (+++), and nine showed less marked, but nonetheless definite lesions (+ and ++). The outstanding feature was the diffuse distribution of the changes. Almost all glomeruli were altered to a greater or lesser extent. In some, the degree of glomerular damage was of about equal severity in all glomeruli, while in others there was considerable variation from one glomerulus to another.

In the kidneys showing the mildest degree of damage, there was glomerular enlargement, and considerable cellular proliferation. Both endothelial and epithelial cells appeared to participate in this. Enlargement of both epithelial and endothelial cells was also striking, the former tending to fill the capsular space, while the latter in many instances almost occluded the glomerular capillaries. The resulting picture was one of reduction in the number of patent glomerular capillary loops. Polymorphonuclear leucocytes were occasionally seen in these glomeruli. Basement membrane changes were only occasionally met with and consisted merely of slight fragmentation of the basement membrane. Homogeneous acidophilic protein material was occasionally seen in Bowman's spaces, and in the tubules, but this was an inconstant feature. Lesions of this type were graded as + and were found in four animals (Fig. 5).

Glomerular lesions of the next grade in severity were characterized by the above changes, which, in the over-all picture, were more marked. Homogeneous acidophilic protein material was seen here with greater frequency, within the glomerular capillary lumina and the capsular space. Cellular proliferation was more marked, and in many of the glomeruli there was fusion of one or more of the glomerular tufts. There was some thickening of the epithelial cells of Bowman's capsules, suggestive of early proliferative changes. Slight thickening and

shredding of the basement membranes into two or three layers could also be seen in some of these glomeruli. Polymorphonuclear leucocytes were seen with about the same frequency as in those of grade +. This type of lesion was graded as ++, and was found in five animals (Fig. 6).

Lesions classed as severe (+++) were seen in two animals. Here there was marked reduction in glomerular capillarity, so that in many glomeruli, only a few patent capillary loops could be seen. Swelling and proliferation of the glomerular endothelial and epithelial cells were marked, and proliferation of the capsular epithelium with beginning crescent formation was commonly encountered. Polymorphonuclear leucocytes were seen in many glomeruli. Thickening and shredding of the glomerular basement membrane was a common feature in these glomeruli, and in many the basement membranes appeared to be discontinuous; *i.e.*, fragmented. This type of change is illustrated in Fig. 7.

The most severe lesions were found in three animals (+++). In these all the above changes were very conspicuous (Figs. 1 and 2). Capsular proliferation was so advanced as to produce well marked epithelial crescents, partial or complete fusion of glomerulus and capsule, and compression of the glomerulus. Proliferative changes within the glomerulus made it impossible to distinguish the individual tufts. Partial or complete obliteration of the glomerular capillaries was conspicuous. This was due in some instances to plugging of the capillary lumina with protein material, and in others to swelling and proliferation of the capillary endothelium. Shredding and fragmentation of both the glomerular and capsular basement membranes were very marked and contributed to the narrowing of the glomerular capillaries in most of these glomeruli (Fig. 3).

Of the four remaining animals in the treated group, two showed no renal lesions, one showed pyelonephritis, and one showed an interstitial nephritis. In both the latter animals, the lesions had none of the characteristics of the diffuse glomerulitis which was ascribed to globulin treatment in the other fourteen animals.

In none of the kidneys of the control animals, nor in the kidneys removed from the treated animals before globulin therapy, was diffuse glomerulitis of the type described above encountered. Lesions of typical, focal, pyelonephritis were seen in kidneys of two of the control animals. Other control animals showed no renal alteration other than the expected changes of hypertrophy (Fig. 4).

It is also of interest that one of the globulin-treated animals showed evidence of renal functional impairment, with the blood urea nitrogen level reaching 187 mg. per cent. At autopsy this animal showed very severe renal damage (Figs. 2 and 3). In the remainder of the animals tested, normal blood urea nitrogen levels were found both before and after treatment.

Focal granulomatous infiltrations were found in the heart valves and valve rings in nine of the globulin-treated animals, and form the basis of a separate report (29). In addition, two animals showed lesions of a proliferative arteritis in the coronary arteries, of the type commonly associated with experimental foreign protein sensitivity.

2. Immunological Studies

It has recently been suggested (23-26, 30) that acute nephritis might be the result of the products of streptococcal infection acting upon renal substance in such a way as to render it antigenic. Antibodies to this renal substance

were thought then to give rise to the lesions of nephritis as a manifestation of local organ-specific, antigen-antibody reaction.

Schwentker and Comploier (23) brought experimental evidence to support such a conception. Using the highly sensitive collodion particle technique they demonstrated antikidney antibodies in the sera of rabbits injected with ground rabbit kidney extract mixed with streptococci. They were unable to show antikidney antibodies when kidney extract or streptococci alone were injected. These findings were confirmed in a series of similar experiments by Cavelti and Cavelti (24-26). Unfortunately the precise relationship between the appearance of antirenal antibodies and the production of renal lesions is not clear in the last mentioned series of articles.

An attempt was therefore made in the present experiments to determine whether antikidney antibodies might play a rôle in the development of the renal lesions in our rabbits. Such investigation seemed important, since in this case, the experimental technique did not involve administration of renal substance, antikidney serum, or streptococci.

Method.—Blood was drawn from ten of the eighteen globulin-treated animals on three occasions: before the first globulin injection, before the desensitizing dose of globulin on the 11th day of the experiment, and finally just before the animals were killed. The blood was centrifuged and the serum drawn off. A simple qualitative ring test technique, similar to that employed by Hawn and Janeway (22) was used in all tests. In this technique a quantity of test antigen is carefully layered over the serum in a small test tube (ours were 3×40 mm.). The tubes were incubated at 37°C . for 1 hour and promptly read. The formation of a clear cut white ring at the interface between the serum and antigen was recorded as positive. Reactions were roughly graded from zero to four plus. Tests were made on these ten sera for the following:—

1. Antibodies to bovine serum gamma globulin. 0.05 per cent globulin solution was used as test antigen.
2. Antibodies to rabbit kidney. The serum of each rabbit was tested against saline extract of perfused ground rabbit kidney. Twenty per cent, 10 per cent, and 2 per cent kidney extracts were used. The kidneys removed from the animals prior to globulin therapy had been washed free of blood by perfusion with normal saline at the time of removal. They were then stored at -20°C . until required for serological testing. The serum of each rabbit was tested then against extract of the rabbit's own kidney, as well as against the pooled extracts of several kidneys.
3. Antibodies to rabbit liver. This served as a control of test 2 above. Twenty per cent, 10 per cent, and 2 per cent extract of ground perfused rabbit liver was used as test antigen.

In addition to these tests, each lot of bovine gamma globulin was tested against the individual and pooled rabbit kidney extracts, and also against rabbit liver extract and sera of all the animals before treatment was initiated. This step was necessary in order to rule out the possible presence of antibodies to rabbit organs and serum in the bovine gamma globulin. All serological tests were controlled by corresponding tests with normal saline.

Intradermal skin sensitivity tests were carried out in all animals just before killing. For this purpose, a shaved area on the back of the animal was injected intradermally with 0.1 cc. of each of the following substances: (a) bovine gamma globulin (10 per cent solution), (b) rabbit kidney extract (20 per cent saline suspension of rabbit's own kidney), (c) rabbit liver

extract (20 per cent saline suspension), and (d) normal saline. In ten of the animals this test was done 2 days before killing, and the tests were read at the time of killing. In the other eight globulin-treated animals the remaining kidney was surgically removed before skin testing. It was hoped thus to eliminate the possibility that the remaining kidney might be absorbing any kidney antibody that may have been present, rendering it undetectable by skin test. Unfortunately these animals failed to survive the second nephrectomy for a long enough period to allow accurate reading of the tests.

Results.—The results of the serological and skin tests, and their relation to the presence of renal lesions are shown in Table I. Preliminary tests showed no demonstrable anti-rabbit-organ antibodies in any of the lots of bovine serum gamma globulin. Sera from eight of the ten animals on which serological studies were done gave moderately strong reactions with bovine globulin 11 days after the initial globulin injection. The reactions of the remaining two animals were weak or doubtful at this time. All the animals showed antibodies to bovine gamma globulin in sera drawn just before killing.

The sera of two animals gave positive reactions with extracts of their own kidneys 11 days after the first globulin injection. One of these animals showed diffuse glomerulitis of the most severe degree at autopsy (Figs. 2 and 3). The other showed rather severe focal pyelonephritis which was quite different morphologically from the lesions in other treated animals, and which was considered spontaneous. Less marked reactions were obtained between the sera of three animals and the pooled kidney extract. However, in only one of these did a corresponding reaction with the rabbit's own kidney appear. All reactions with both liver and kidney extracts were negative in sera drawn just before the animals were killed.

All animals skin-tested showed some degree of skin sensitivity to bovine gamma globulin. These tests were graded according to severity. The most marked reactions were classed as ++++, and consisted of well marked areas of edema, erythema, and central necrosis. Reactions graded as + consisted of a zone of edema only. Some slight reaction was noted at the site of injection with kidney extract in three animals. These reactions consisted of edema only. Two of these three animals had histologically normal kidneys at autopsy, whilst the third showed lesions of diffuse glomerulonephritis.

In general, then, it may be stated that no correlation could be noted between the results of immunological tests for antikidney antibodies done in this experiment, and the presence or severity of renal lesions. It should also be pointed out that although all animals showed antibodies to the injected antigen (bovine gamma globulin), in four of them the lesions of diffuse glomerulonephritis were lacking.

3. Blood Coagulation Studies

A frequent observation in both human and experimental nephritis in the acute stage, is the presence of fibrin or protein coagula in the glomerular capil-

TABLE I
Relation of Immunologic Reactions to Presence of Renal Lesions

Animal No.	Time of test	Serologic reaction to				Intradermal test			Glomerulonephritis
		Own kidney extract	Pooled kidney extract	Liver extract	Globulin	Kidney extract	Liver extract	Globulin	
2	Control*	0	0	0	0				0
	11 days	0	+	0	++				
	17 days	0	0	0	++	+	0	++	
4	Control	0	0	0	0				++++
	11 days	++	+	0	++				
	17 days	0	0	0	++	0	0	+++	
5	Control	0	0	0	0				Focal interstitial nephritis only
	11 days	++	0	0	++				
	17 days	0	0	0	++	0	0	++	
7	Control	0	0	0	0				++
	11 days	0	0	0	++				
	17 days	0	0	0	++++	+	0	++	
8	Control	0	0	0	0				++
	11 days	0	+	0	++				
	17 days	0	0	0	++++	0	0	++	
9	Control	0	0	0	0				++
	11 days	0	0	0	+				
	17 days	0	0	0	++	0	0	+	
11	Control	0	0	0	0				Spontaneous pyelonephritis only
	11 days	0	0	0	++				
	17 days	0	0	0	++	0	0	+	
12	Control	0	0	0	0				+++
	11 days	0	0	0	++				
	17 days	0	0	0	++	0	0	++++	
13	Control	0	0	0	0				0
	11 days	0	0	0	±				
	17 days	0	0	0	++	+	0	+	
14	Control	0	0	0	0				++++
	11 days	0	0	0	++				
	17 days	0	0	0	++++	0	0	+	

* Sera obtained 24 hours before initial globulin injection.

laries (31-33). Since this phenomenon is one of the earliest morphologically detectable changes in nephritis, it was thought that some alteration of blood coagulability might be found during the period in which nephritis could be presumed to be developing. In this connection, Silfverskiöld (34) was able to prevent, by preliminary injection with heparin, the urinary changes of experimental horse serum nephritis in rabbits.

Morphological changes in the kidneys of these animals are only briefly dealt with by Silfverskiöld, but he states that "the kidneys of the heparinized animals seemed to be less involved than the non-heparinized controls." It is interesting that in a similar experiment by Silfverskiöld with nephrotoxic nephritis in rats, heparin failed to have this effect. Silfverskiöld's work has not been re-

TABLE II

Changes in the Blood Coagulation Time during the First 7 Days after a Massive Bovine Gamma Globulin Injection

	No. of readings	Mean coagulation time	Standard deviation
		min.	
18 globulin-treated animals	168	3.90 ± 0.104	1.35
18 nephrectomized controls*	34	5.42 ± 0.215	1.25
5 saline-injected controls†	50	5.68 ± 0.16	1.16

* These observations were made on the same 18 animals that were subsequently injected with globulin. They were recorded 3 to 6 weeks after unilateral nephrectomy, within the 5 days preceding the initial globulin injection.

† These observations were made during 7 days following injection with 10 cc. of normal saline per kilo of body weight.

peated, and examination of blood coagulability in experimental nephritis does not appear to have been carried out before.

Method.—Coagulation time observations were made on globulin-treated and control animals. The capillary tube technique was used in all determinations, and times were recorded to the nearest 1/10th of a minute. In this technique blood from a fresh cut in a small ear vein was drawn into a glass tube of capillary bore. The tube was mounted upright in a piece of plasticine, and small bits of it broken off every few seconds. The coagulation time was recorded as the time at which, when the tube was broken, a thin strand of clot pulled away with the broken end. Daily observations were made in most instances, and hourly recordings were made on several occasions. Estimation of the normal coagulation time was based on thirty-four observations made on the eighteen animals which were subsequently injected with globulin.

Results.—The results of the coagulation time observations on globulin-treated and control animals are shown in Table II. The individual variation in the readings was such that there was no uniformity of coagulation time in one particular animal from day to day, nor in all animals on any particular

day of the experiment. The results do indicate, however, that during the 1st week after globulin injection there is an increase in blood coagulability, as shown by a shortening of the mean coagulation time during this period. This change was not noted during the 6 days following the second globulin injection.

The normal mean coagulation time of the eighteen animals, as estimated from determinations made within the 5 days immediately before the initial globulin injection, was 5.42 minutes, with a standard deviation of 1.25. The mean coagulation time of the animals during the first 7 days after the first globulin injection was only 3.90 minutes, with a standard deviation of 1.35. Analysis of this difference in mean coagulation times shows that the increase in blood coagulability is statistically significant. During the 6 days following the second globulin injection the mean coagulation time did not differ significantly from the normal time, the mean being 5.39 minutes. No significant alteration in blood coagulability was observed in the five control animals on which coagulation studies were done following injection with normal saline (see Table II).

On the 8th day after the initial globulin injection, eight of the eighteen animals showed a slight prolongation of coagulation time (to 7.5-12 minutes). Although it was considered that this might be a phenomenon associated with the disappearance of antigen from the blood stream (as was shown by Hawn and Janeway to occur at about this time), further observations will have to be made to confirm this incidental finding. Several of the animals also showed marked prolongation of coagulation time within $\frac{1}{2}$ hour following the desensitizing injection of globulin on the 11th day of the experiment. Absence of this well known transient feature of anaphylaxis (35) in the other animals was probably due to the fact that all observations on this date were not made at exactly the same interval after globulin injection.

DISCUSSION

There can be little doubt that the high incidence of diffuse glomerulitis in our animals was the result of their treatment with bovine gamma globulin. The experimental injury does not resemble any spontaneous renal lesion in rabbits, and similar lesions did not occur in any of the control animals. Preliminary nephrectomy, together with the use of two massive globulin injections, appears to have markedly increased the severity of the nephritis in comparison to that produced by Hawn and Janeway (22) with a single injection in the intact animal. The morphological appearance of the lesions closely resembles that of human glomerulonephritis. The features of glomerular enlargement, cellular proliferation, shredding of basement membranes, protein deposits in the glomerular capillaries and Bowman's spaces, and the more advanced changes of crescent formation, and fusion of the glomerular tufts are characteristics common to these experimental lesions and those of human

glomerulonephritis. A further similarity is the diffuse involvement of practically all glomeruli in both instances. The appearance of the lesions in the more severely damaged kidneys is such that chronicity and progression of the pathological changes in the absence of further treatment might be presumed (Figs. 1, 2, and 3). This constitutes an added feature common to human nephritis and experimental globulin nephritis. The total morphological picture in the kidneys of these animals appears, in fact, identical with that of human diffuse glomerulonephritis in its acute and subacute phases.

The experimental method employed in these experiments appears to us to be equal or superior to other techniques in its capacity to produce in high incidence, a close counterpart of human diffuse glomerulonephritis. It should be possible, through study of the various features of globulin nephritis and their integration with those of other varieties of experimental nephritis and the human disease, to draw some conclusion regarding the etiology and pathogenesis of the lesions.

The currently favored hypotheses of the etiology and pathogenesis of nephritis assign a fundamental rôle to the state of hypersensitivity (23-26, 30). In the human disease the association between hypersensitivity and the development of nephritis is suggested by the time relation between the onset of an upper respiratory infection (usually streptococcal) and the appearance of renal symptoms. In the various forms of experimental glomerulonephritis which can be said to resemble the human disease, hypersensitivity invariably accompanies the development of the lesions. The mechanism by which hypersensitivity produces nephritis has been the stumbling block in most theories causally relating the two conditions.

Recently Cavelti and Cavelti (24-26) demonstrated experimentally that kidney tissue can be rendered antigenic for homologous species by the addition of streptococci to an extract of perfused, ground kidney. This had been shown earlier by Schwentker and Comploier (23) who, in addition, demonstrated antibodies to kidney in sera of human nephritics. In both these pieces of work, the use of the highly sensitive collodion particle agglutination technique was necessary for consistent demonstration of kidney autoantibodies. These results suggested to the Caveltis and to Schwentker and Comploier that in streptococcal infection, the bacteria or their products may act upon the kidney in such a way as to render it antigenic, and that nephritis ensues as the result of the action of antikidney antibodies on renal tissue *in situ*.

In our experiments neither kidney antibodies, streptococci, nor renal substance was injected. Nevertheless, the resulting morphological picture was indistinguishable from that produced by one or another of these agents. It seems reasonable to assume, therefore, that the mechanism by which the lesions were produced must also be similar, if not identical. It was not, however, possible to demonstrate that the development of nephritis in this instance was mediated, or even accompanied by the formation of antikidney

antibodies. While the simple ring test and intradermal sensitivity tests used here are certainly less sensitive than the collodion particle agglutination technique used by the above investigators, they are also less likely to give non-specific cross-reactions.

These results seem to cast some doubt upon the kidney autoantibody hypothesis, in the pathogenesis of this type of nephritis. Indeed, this entire concept is brought into question by the recent publication of Humphrey (36). Using the Cavelti technique, he was unable to reproduce their results, either morphologically or immunologically, in a single animal.

It is more difficult to assess the rôle of hypersensitivity in general in the development of globulin nephritis. Antibodies to the injected globulin were demonstrated in all animals tested. Hypersensitivity can thus be said to have accompanied the development of the renal lesions in every instance. However, critical consideration of the evidence from this experiment and that of others makes it clear that no cause-and-effect relationship between hypersensitivity and nephritis has been unequivocally established. In view of this, it seemed advisable to examine other possible factors. One such factor which was investigated was that of possible changes in blood coagulability during the time of development of globulin nephritis. There was, indeed, a significant increase in blood coagulability during the first 7 days after the initial globulin injection. Moreover, the appearance of the more severe renal lesions in these rabbits is consistent with their origin at about this time. This alteration in coagulability was inconstant from one animal to another, and in the same animal from day to day, but showed up clearly as a depression of the mean coagulation time in the treated animals during the first 7 days of treatment. Although the changes in individual animals were not such as to allow us to predict which of them would show renal lesions, the alteration does constitute evidence of a physicochemical alteration in the blood following massive globulin injection. It is possible that the formation of fibrin thrombi in the glomerular capillaries may be related to an alteration of blood coagulability. The work of Silfverskiöld cited above, indicating an amelioration of one type of experimental nephritis by heparin, adds some support to this suggestion. There is sufficient evidence from both Silfverskiöld's work and our own to justify closer examination this possibility. It seems equally possible that the glomerular capillary plugs are protein coagula resulting from a decrease in the colloidal suspension stability of the plasma protein molecules. For instance, it is known that hyperglobulinemia is associated with an increase in red blood cell sedimentation velocity. It seems possible that a large quantity of globulin may produce physicochemical alteration sufficient to bring about precipitation of protein in the glomerular capillaries where the blood is concentrated about 25 per cent through fluid loss. It seems essential to consider such possibilities in experiments like the present, in which large amounts of

protein are injected, before more complex mechanisms are accepted as the basis of the lesions produced.

It should be emphasized that although the most striking morphological lesion produced by massive dosage with bovine gamma globulin is a diffuse glomerulitis, the injection of purified antigen did not in our experiments produce this single type of lesion alone. Nine of the globulin-treated animals showed focal granulomatous lesions in the heart valves and valve rings, and two showed coronary arteritis (29). It should be further noted that cardiac and renal lesions did not always coexist in the same animals. This confirms the findings of Hawn and Janeway (22), who also found both cardiac and renal lesions in their globulin-treated animals. It appears that if the lesions of focal cardiac granulomata and diffuse glomerulonephritis are based upon hypersensitivity, that the specificity of the antigen is not the important factor in the production of one lesion or the other.

SUMMARY AND CONCLUSIONS

A high incidence of acute diffuse glomerulitis was produced in unilaterally nephrectomized rabbits by injection with two successive doses of purified bovine serum gamma globulin (fraction II). This experimental nephritis is morphologically analogous to human acute and subacute diffuse glomerulonephritis. The technique described is advanced as a valuable experimental method in the study of the pathogenesis of glomerulonephritis.

Qualitative immunological investigations produced no evidence that the pathogenesis of experimental globulin nephritis is mediated by kidney auto-antibodies. The rôle of hypersensitivity in the pathogenesis of nephritis is discussed.

During the 1st week of the development of experimental globulin nephritis there is a significant increase in blood coagulability, as shown by a lowering of the mean coagulation time in globulin-treated animals during this period. This observation has not been reported previously. The possible relation of this increased blood coagulability to the formation of coagula in the glomerular capillaries is discussed.

The injection of a single purified antigen (bovine serum gamma globulin) produced three distinct types of lesion, diffuse glomerulitis, focal granulomata of the heart valves and valve rings, and coronary arteritis.

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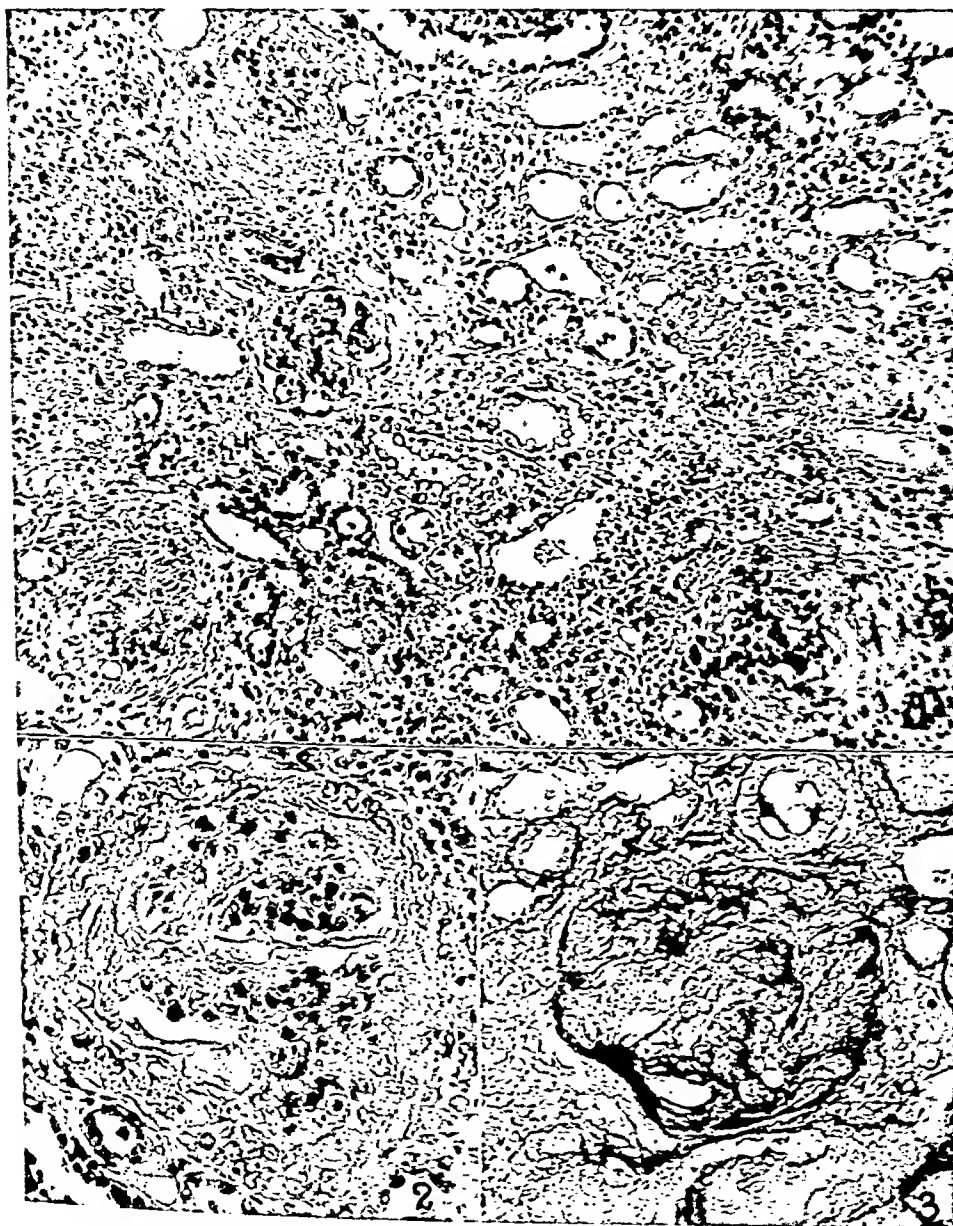
EXPLANATION OF PLATES

PLATE 22

FIG. 1. Diffuse glomerulonephritis, grade + + + + in animal 4 which had uremia. The diffuse distribution of the lesions is shown. In addition to marked proliferative changes in four glomeruli, a diffuse increase in interstitial connective tissue, atrophy of tubules, and tubular casts are to be seen. Hematoxylin and eosin. $\times 137$.

FIG. 2. Diffuse glomerulonephritis, grade + + + +; glomerulus from same kidney as shown in Fig. 1. There is marked cellular proliferation, forming almost complete synechia between glomerulus and capsule, and compressing remnants of glomerular tuft at center. A protein coagulum is seen near the center. Hematoxylin and eosin. $\times 300$.

FIG. 3. Diffuse glomerulonephritis, grade + + + +; same kidney as in Fig. 1 and 2. There is irregular thickening and shredding of both glomerular and capsular basement membranes. Mallory-Haidenhain. $\times 300$.



(More and Waugh: Globulin glomerulonephritis in rabbits)

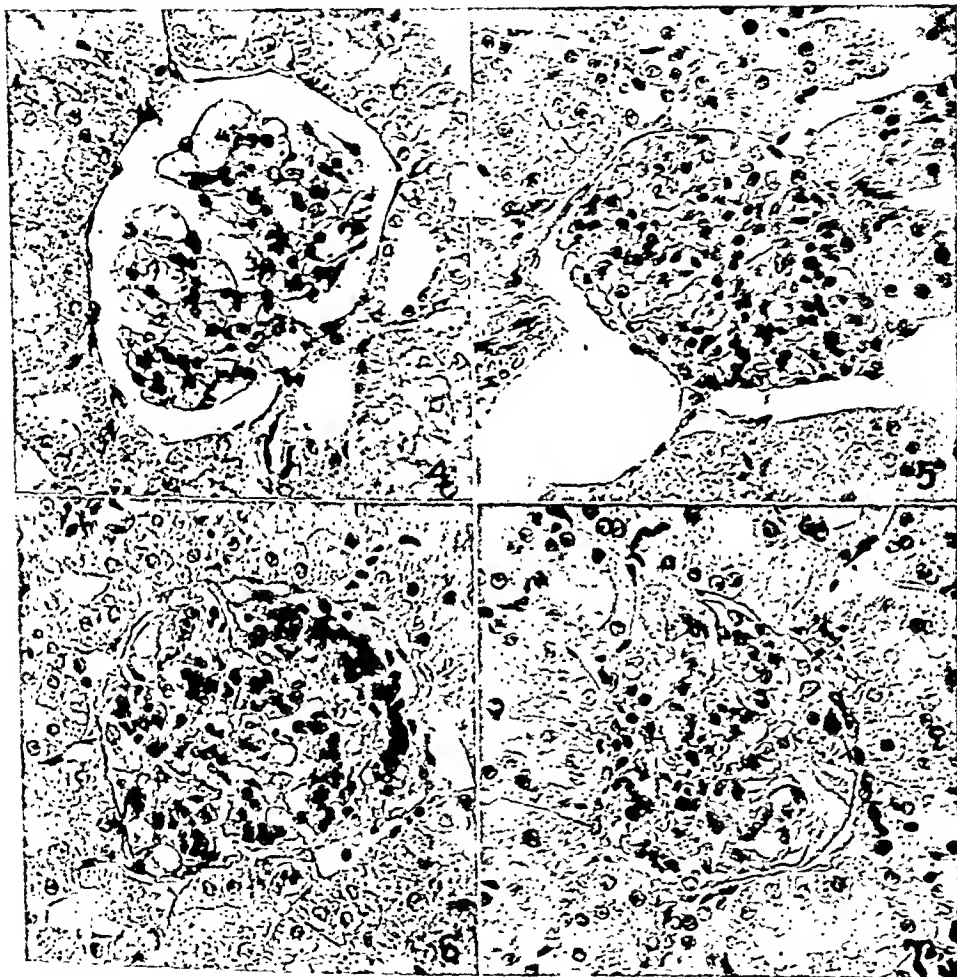
PLATE 23

FIG. 4. Normal glomerulus from nephrectomized control animal. Glomerular tufts are discrete, and capillary loops delicate and patent. Hematoxylin and eosin. $\times 300$.

FIG. 5. Diffuse glomerulonephritis, grade +. There is conspicuous increase in glomerular cellularity and reduction in size of capillary lumina. The more darkly staining nuclei are those of endothelial cells. Hematoxylin and eosin. $\times 300$.

FIG. 6. Diffuse glomerulonephritis, grade ++. Here the reduction in the capillary lumina is more marked. This is due to proliferation and swelling of endothelial cells into the capillaries. Thickening of the epithelium of Bowman's capsule suggests early crescent formation. Hematoxylin and eosin. $\times 300$.

FIG. 7. Diffuse glomerulonephritis, grade ++++. Proliferative changes have led to early crescent formation. Hyalin protein exudate in Bowman's space. Hematoxylin and eosin. $\times 300$.



(More and Waugh: Globulin glomerulonephritis in rabbits)

CARDIAC LESIONS PRODUCED IN RABBITS BY MASSIVE INJECTIONS OF BOVINE SERUM GAMMA GLOBULIN*

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PLATES 24 TO 26

(Received for publication, February 7, 1949)

In a series of experiments designed to study the lesions of glomerulonephritis produced in rabbits by repeated intravenous injections of bovine serum gamma globulin¹ (7), a high incidence of granulomatous lesions of the heart valves and valve rings was noted. This was deemed of sufficient interest to report.

Recent interest in lesions of the heart produced in animals, anaphylactically sensitized to foreign proteins, has been stimulated by their reported resemblance to the cardiac lesions of human rheumatic fever (1, 2, 4, 5, 10). The highest incidence of such lesions has been reported by Rich and Gregory (1, 2). However, in a similar but more extensive experiment performed by More and McLean (5) in this Institute, the difficulty of interpreting such lesions was emphasized.

In all the experiments cited above, normal horse serum was employed as the antigen. Further work along these lines has been done by Hawn and Janeway (6) using purified bovine gamma globulin with the reported production of heart valve and valve ring lesions in 20 per cent of their animals. However, they did not emphasize these lesions and gave attention particularly to the nephritis that also appeared in their globulin-treated animals.

In our experiments, the use of massive intravenous injections of globulin resulted in the highest incidence of valvular and valve ring lesions thus far reported as produced by the use of any foreign protein.

Experimental Procedure

The treated animals consisted of a group of seventeen albino rabbits, weighing approximately 2 kilos each, obtained from various dealers. They were first unilaterally nephrectomized. After an interval sufficient for the animals to recover from the operation, they were injected intravenously with purified bovine gamma globulin (fraction II) in a dose of 1 gm. per kilo of body weight in a 10 per cent solution in normal saline. On the 11th day a de-

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‡ The purified bovine gamma globulin (fraction II) used in these experiments was supplied through the courtesy of Lawrence L. Lachat of the Biochemical Sales Research Department, Armour and Company, Chicago.

sensitizing dose of 10 cc. of 1 per cent globulin in normal saline was given slowly intravenously. On the 12th day the animals were again given an intravenous injection of 1 gm. of globulin per kilo in a 10 per cent solution in normal saline. They were killed 18 days after the first injection. The hearts were fixed in Bouin's and Helly's fluids and cut transversely through the base in such a way as to present a large area of mitral and tricuspid valves and valve rings for histological study. The aortic and pulmonic valves did not appear in the sections with regularity. Sections were stained with hematoxylin and eosin; hematin, phloxine, and saffron; Weigert's elastic tissue stain and Mallory's phosphotungstic acid hematoxylin.

Seven control animals were unilaterally nephrectomized and allowed to live for intervals varying from 3 weeks to 7 months. Four of these animals were injected with normal saline, equivalent in volume to the dose of globulin administered to the treated animals; that is, 10 cc. per kilo. They were otherwise handled in the same way as the treated group.

Nine of the treated animals were skin-tested by an intradermal injection of 0.1 cc. of 10 per cent globulin 2 days before they were killed. The sera of these animals were tested for the presence of antibodies to globulin by a qualitative ring test on three occasions: the day before the initial injection; the 11th day (the day before the desensitizing dose); and immediately before the animals were killed.

RESULTS

Focal granulomatous lesions were found in valves and valve rings of nine of the globulin-treated animals, constituting 52 per cent of the group.

Valve lesions were present in eight animals, or 47 per cent. Lesions of the valve rings were present in four animals, or 23 per cent. Lesions in both locations were present in three animals. No similar lesions were present in the controls. The valve lesions were seen on more than one, and in some instances on all the valve cusps visible in the sections. The valve ring lesions were seen only singly in each case.

The valve lesions were focal in type and consisted of a raised cellular nodule (Fig. 1), causing an increase in the thickness of the valve. Sometimes the whole thickness of the valve was involved and the excrescences projected on both sides (Figs. 2 and 3). In lesions regarded as early (Fig. 1) the superficial portion consisted of a row of plump cuboidal endothelial cells closely lined up beside one another overlying edematous connective tissue in which there was an increase in fibroblasts, and large mononuclear cells with a few scattered lymphocytes. In more pronounced lesions the surface consisted of a thick layer of what were apparently proliferated endothelial cells containing a moderate number of mitoses overlying a rather dense accumulation of large mononuclear cells. Mixed with these cells were a few lymphocytes and polymorphonuclear leucocytes (Fig. 3). The large mononuclear cells of the deeper layers had large vesicular nuclei with finely divided chromatin and several variable and centrally placed masses of chromatin. The cytoplasm was abundant, moderately basophilic, finely granular, and had a sharp cellular border. Also present were cells with similar cytoplasm containing 3 to 8 nuclei. The rather similar appearance of the multinucleated cells and the mononuclear forms suggested an origin of the giant cells from the large mononuclear cells. In one of the lesions the frequency of mitoses in these cells was striking and young fibroblasts were prominent (Fig. 4). The connective tissue of the valves affected was edematous and close to the lesions the collagen fibers themselves appeared swollen, and somewhat homogeneous and a few small areas showed fragmentation. These areas of collagen degeneration did not present the appearance of fibrinoid necrosis with the stains employed (Fig. 3). Sur-

rounding the lesions, but not generally included in them, was a prominent number of cells resembling the so called myocyte of Anitschkow.

The lesions of the valve rings were found in four animals. These consisted of localized granulomatous formations with a dense accumulation of large mononuclear cells similar to those seen in the valves. Multinucleated giant cells were prominent in one instance (Fig. 6) and in this animal young fibroblasts and a few capillaries were also present. The connective tissue of the valve rings was altered in a similar way to that of the valves. There was localized disorganization of the normal architecture and replacement by the cellular components and edematous matrix just described (Fig. 6). Mitoses were frequent in the lesions and also present in the connective tissue cells surrounding the lesions. In many places, the endothelium at the base of the valves showed an even more marked hyperplasia than that seen in the valves (Fig. 5).

In the myocardium the lesions encountered consisted of a focal myocarditis. Such lesions were found in the control as well as in the experimental group, the most pronounced of these occurring in one of the control animals. These resembled the lesions described as spontaneously occurring in rabbits by Miller (8). At the same time they were very similar to the lesions ascribed to treatment with foreign proteins by previous workers (1, 2, 4, 6).

Lesions of the endocardium essentially similar to those in the valves were seen in two treated animals. These were regarded as identical with some of the lesions described by Miller (8) as spontaneous and are thus not interpreted as clearly due to the treatment. However, it should be noted that none of these was present in controls and that the lesions were associated with those of valves and/or valve rings in both cases.

Lesions of arteries were seen in the hearts of two animals. They consisted of lesions which have been described as the sclerotic type of coronary arteritis due to foreign protein hypersensitivity (4) or as healed or healing lesions (3). Necrosis of the arterial wall was only seen in one instance.

A positive Arthus reaction to globulin was obtained in all the animals tested immediately prior to killing. Precipitins to the globulin antigen were demonstrated in the blood of the nine animals whose sera were tested by means of ring tests before the second injection and just before the animals were killed (7).

DISCUSSION

The incidence of valve and valve ring lesions in our animals is the highest thus far reported in animals sensitized to foreign protein. Furthermore, the statistical incidence of the lesions is such that there can be no doubt that the lesions were the result of treatment. The latter does not appear to be the case in other reported work in this field where there has been no clear separation of the incidence of the valvular and valve ring lesions from that of the equivocal myocardial lesions.

The most striking parallel between the lesions produced in our animals and the lesions of human rheumatic fever is their location in valves, valve rings, and subvalvular angles; moreover, when only one surface was involved the lesions of the valves appeared on the auricular surface (Fig. 1). Histologically, their structure resembles the lesions of human rheumatic fever in that

they are peculiar focal granulomatous lesions which include the features of collagen damage, mononuclear cell infiltration, giant cell formation, and endothelial proliferation. The last was particularly prominent in the sub-valvular angle, a common finding in human rheumatic carditis. However, the amorphous deposits overlying the granulomatous lesions which form the verrucose vegetations in the human valvulitis were not seen in any of our animals. The mononuclear cells and giant cells of the experimental lesions were not identical with the Aschoff cells of human rheumatic fever, and the collagen damage was not so striking. Lesions of the myocardium similar to those reported by other workers as resembling Aschoff bodies (1, 2, 4) were found in our animals. However, we were unable to interpret them as corresponding to the lesions of rheumatic fever due to their resemblance to spontaneous lesions of the myocardium occurring in rabbits (8) and to those occurring as a result of epizootic infections (9). These lesions of the myocardium were present in both the treated and control animals and could thus not be ascribed to treatment. Therefore, although the cardiac lesions of man have not been reproduced in every detail in the hearts of our experimental animals, we do believe that the similarities between the experimental and human valve and valve ring lesions indicate that they may well have a similar pathogenesis.

The observed discrepancies may be no more than might be accounted for by the well recognized variation in the tissue response of different species. It therefore seems justifiable to pursue this general experimental approach in attempting to elucidate the etiology and pathogenesis of human rheumatic fever.

The galaxy of lesions produced by anaphylactic sensitization to the mixture of antigens contained in horse serum has led to the speculation that there might be a clear cut relationship between one specific type of lesion and a given purified antigen. The use of purified serum gamma globulin by us and of this and other purified antigens by Hawn and Janeway does not support this concept. In our animals intravenous injection of purified bovine serum gamma globulin gave rise to a glomerulonephritis (7) and in almost equal incidence, a granulomatous inflammation of heart valves and valve rings. Also produced, though in a much smaller number of animals, was an arteritis.

The most important etiological factor in producing lesions in anaphylactically sensitized rabbits is widely considered to be the hypersensitive state. That hypersensitivity existed in our tested animals is indicated by the presence of a positive Arthus reaction and precipitins in the blood (7). However, the coexistence of hypersensitivity and tissue lesions does not, by itself, necessarily demonstrate a pathogenetic relationship between the two. It is thus obvious that further study is necessary to establish the relationship of hypersensitivity to the production of tissue lesions in animals and in rheumatic fever in man.

CONCLUSIONS

Granulomatous lesions of the valves and valve rings have been produced in 52 per cent of rabbits treated by massive intravenous administrations of bovine gamma globulin in such a way as to establish a state of anaphylactic hypersensitivity in the animals. The incidence of the lesions is greater than has heretofore been reported as produced by the use of any type of foreign protein injection in rabbits.

The use of a purified protein antigen has resulted in the production of several different types of lesions; namely, a granulomatous inflammation of heart valves and valve rings, a glomerulonephritis, and an arteritis.

The lesions of the heart cannot be regarded as being identical with the lesions of human rheumatic fever, but they are considered to be sufficiently similar to warrant a continuation of this approach to the study of the etiology and pathogenesis of rheumatic fever in man.

We are indebted to the members of the staff of the Department of Bacteriology and Immunology, McGill University, whose advice and facilities were generously placed at our disposal.

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EXPLANATION OF PLATES

PLATE 24

FIG. 1. Heart valve showing nodular excrescence on auricular surface. There is hypertrophy and proliferation of endothelium overlying a localized area of edema and loss of structure of the normally loose collagen. An increased content of large mononuclear cells and a few lymphocytes and polymorphonuclear cells are also visible. (Rabbit 5.) Hematoxylin and eosin. $\times 400$.

FIG. 2. Heart valve of rabbit 8 showing topography of localized lesion. Marked cellular thickening of the valve is visible. Hematoxylin and eosin. $\times 115$.



(More *et al.*: Globulin endocarditis in rabbits)

PLATE 25

FIG. 3. Higher magnification of a portion of the valve shown in Fig. 2. Edema and alteration of the collagen are evident in both areas of infiltration. Endothelial proliferation and infiltration of large mononuclear cells, lymphocytes, and the occasional polymorphonuclear cell are visible. Hematoxylin and eosin. $\times 310$.

FIG. 4. Lesion of valve near base showing endothelial proliferation, large mononuclear cells, giant cells, and numerous young fibroblasts in an edematous stroma. The lesion is surrounded by a sprinkling of lymphocytes and "Anitschkow" cells are visible outside the lesion. (Rabbit 4.) Mitoses are numerous in the lesion (see insert). Hematoxylin and eosin. $\times 250$. Insert. $\times 900$.

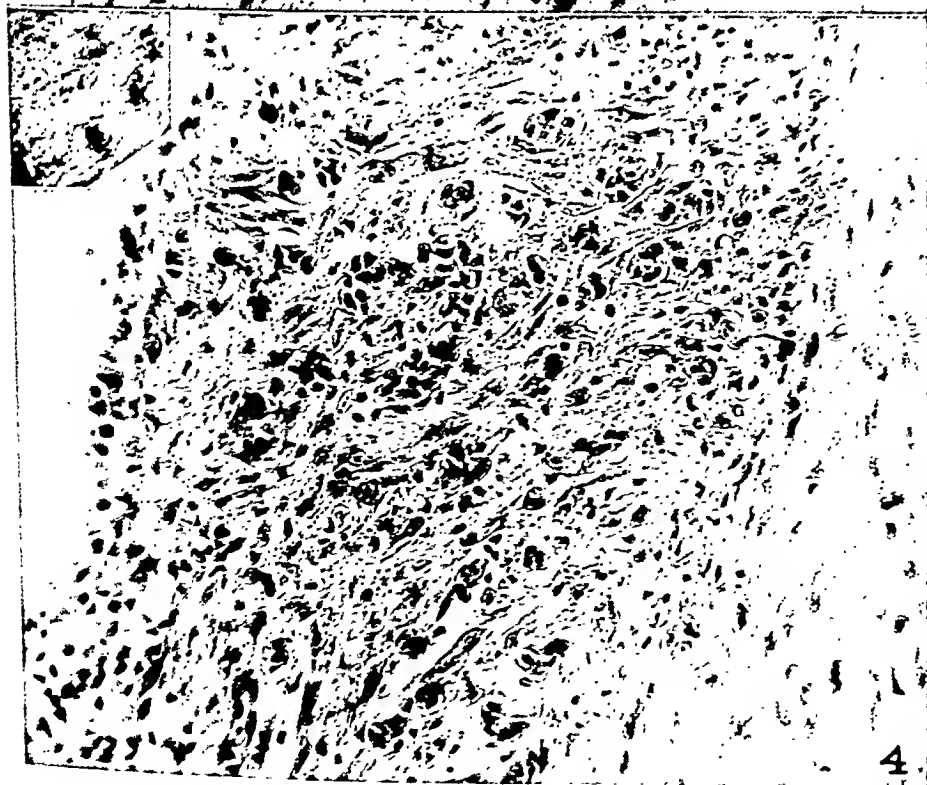
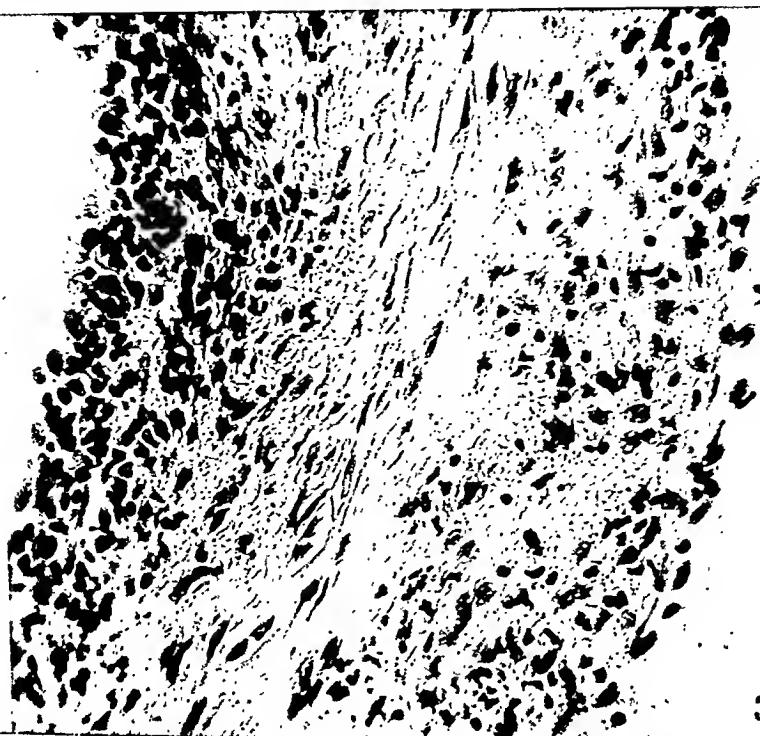


PLATE 26

FIG. 5. Base of valve showing marked endothelial proliferation of subvalvular angle. (Rabbit 14.) Hematoxylin and eosin. $\times 126$.

FIG. 6. Lesion of valve ring (rabbit 4) showing the disorganized and edematous ground substance, prominent multinucleated giant cells, young fibroblasts, and capillaries. Mitoses in cells at the periphery and surrounding the lesion were easily seen under the microscope. Hematin, phloxine, and saffron. $\times 350$.



(More *et al.*: Globulin endocarditis in rabbits)

RESPIRATION OF TYPHUS RICKETTSIAE*

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(Received for publication, February 10, 1949)

Studies yielding information concerning the growth or metabolism of obligate intracellular parasites should be of obvious theoretical and possibly practical importance. Rickettsiae, a group of intracellular parasites which appear to be physiological as well as morphological intermediates between the viruses and bacteria, seem to offer a favorable group for such studies. The experiments reported below indicate that this indeed may be the case.

Methods

Three strains of typhus rickettsiae were used in this study: the Breinl strain of epidemic typhus, the Madrid E strain of epidemic typhus (3, 5) and the Wilmington strain of murine typhus. The three strains were maintained by serial passage in embryonated eggs (4).

Preparation of Rickettsial Suspensions.—Suspensions of typhus rickettsiae were prepared from infected yolk sac pools which were homogenized in a Waring blender with 1 volume of a buffered isotonic salt solution and quickly shell frozen in an alcohol-dry ice mixture. The composition of the salt solution is described below. A portion of the 50 per cent yolk sac suspension was thawed, diluted with $2\frac{1}{2}$ volumes of the same salt solution, and centrifuged at 5000 R.P.M. in an angle centrifuge for 45 minutes. The supernatant was discarded; the precipitate was resuspended to the same volume, treated with 1 gm. of celite (6, 12) for each 6 gm. of yolk sac, and centrifuged at 1000 R.P.M. for 30 minutes to remove cell fragments. The supernatant was again centrifuged at 5000 R.P.M. and the precipitate resuspended to the desired volume, usually equal to one-half that of the original yolk sac. This suspension was centrifuged at 500 R.P.M. for 10 minutes to remove any remaining particles; the supernatant turbid fluid constituted the final suspension which was used for measurements of respiration. It generally contained about 50 per cent of the rickettsiae originally present as estimated by its toxicity for mice. The protein nitrogen content was around 0.5 to 1 mg. N per ml., but varied somewhat with each preparation. All procedures were carried out at 0–5°C. and the suspensions were used immediately after preparation.

The salt solution for washing the rickettsiae consisted of 0.122 M KCl; 0.0074 M NaCl; 0.0041 M KH_2PO_4 ; and 0.0078 M Na_2HPO_4 ; pH 7.0. In many instances 0.04 per cent casein hydrolysate or 0.0045 M potassium glutamate was also present, except in the solution used for resuspension of the final precipitate. More recently respiration measurements have

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been carried out at pH 7.5 rather than at pH 7.0 as the oxygen uptake is greater at the higher pH. In such cases the final precipitate was resuspended in a solution of the following composition: 0.126 M KCl; 0.0018 M NaCl; 0.0012 M KH_2PO_4 ; 0.0106 M Na_2HPO_4 ; pH 7.5.

Oxygen Consumption Measurements.—These were carried out by the conventional Warburg method at 34.3°C. The reaction mixture consisted of 1.5 ml. of a rickettsial suspension; 0.2 ml. of a solution of 0.012 M MgCl_2 and 0.004 M MnCl_2 ; substrate, neutralized with KOH, at the indicated concentration, and salt solution of the indicated pH to bring the total volume in the vessel to 2.4 ml. The center well contained 0.1 ml. of 10 per cent KOH. Readings were taken at intervals for 3 to 4 hours but the rates given in Table I were those observed for the first 2 hours.

Glucose concentration was determined by the method of Nelson (9), and pyruvate as described by Lardy (7). Toxicity of the rickettsial suspensions for white mice was estimated by the intravenous injection of 0.25 ml. of serial threefold dilutions of the rickettsial suspensions using 4 mice for each dilution (2). Deaths were counted after 24 hours and the dilution of the final rickettsial suspension required to kill 50 per cent of the mice was estimated by the method of Reed and Muench (11). The infectivity of the epidemic typhus preparations was estimated in cotton rats in the manner described in a previous report (8).

RESULTS

Table I contains the data of the experiments which show that partially purified rickettsial preparations from yolk sac infected with *R. prowazeki*, strain E, had a definite oxygen consumption with casein hydrolysate as substrate. The product of similar preparations from normal yolk sac had an insignificant oxygen uptake under the same conditions indicating that the observed metabolic activity was a property of the rickettsiae themselves. This conclusion was further confirmed by the observation that the oxygen uptake of rickettsial preparations from various pools of strain E was directly related to the concentration of viable rickettsiae as determined by two accepted assays for rickettsiae, namely toxicity for white mice and immunization end-point in cotton rats. The direct proportionality which exists between the rate of oxygen uptake and toxicity for white mice is shown by the constancy of the ratio between these two values given in columns (k) and (l) of the table. Furthermore, this same correlation of oxygen uptake with the concentration of viable rickettsiae occurred with the Breinl strain of *R. prowazeki* and the Wilmington strain of *R. mooseri*.

The chief constituent of casein hydrolysate responsible for the observed oxygen uptake is probably glutamic acid since this amino acid brings about an oxygen uptake equal to or, usually, greater than that in the presence of casein hydrolysate (see columns (e) and (f) in the table). Not all of the amino acids have yet been tried, but to date none has been found other than glutamic acid that leads to an increased oxygen uptake by rickettsiae. Carbon dioxide is also produced from glutamic acid, but the R.Q. of 0.85 indicates that the oxidation is incomplete.

No other substrate has been found that is oxidized as rapidly as glutamic acid. It was surprising to find that not only is there no oxygen uptake with

TABLE I
Oxygen Uptake by Purified Preparations of *Typhus Rickettsiae*

Rickettsiae		pH	Rate of oxygen uptake					Toxicity for mice* LD ₅₀	$k_C = \frac{e}{j\ddagger}$	$k_G = \frac{f}{j\ddagger}$
Strain	Pool No.		No substrate	Casein hydrolysate 0.3 per cent	Glutamate 0.0125 M	Pyruvate 0.004 M	Succinate 0.0125 M			
(a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)	(i)	(j)	(k)
microliters O ₂ /hr./ml. rickettsial suspension										
Madrid E	2958§	7.0		5.5			11.5	13	0.42	
Madrid E	2955	7.0	1.9	10.6		4.2	6.4	20	0.52	
Madrid E	2937	7.0		21.1		5.5	19.1	55	0.38	
Madrid E	2956§	7.0	2.3	24.1		8.9	8.9	50	0.48	
Madrid E	3006	7.0		10.4	10.8			17.2	0.60	0.63
Madrid E	3006	7.4		15.7	20.8					
BreinI	3040G	7.0		45.3	61.8	8.1		100	0.45	0.62
BreinI	3040E	7.0			96.4		20.4	172		0.56
BreinI	3040	7.4			107.0	14.1	18.1			
BreinI	3040, frozen	7.4			10.0	5.6	39.2			
Wilmington	3096	7.4			28.0	2.9	3.5	55		0.51
Wilmington	3097	7.4			54.5	5.3	4.6	100		0.54
Wilmington	2676	7.4	0.7		87.7	6.8	6.9	172		0.51
Wilmington	2676, frozen	7.4			5.5	0.0	23.1	19		0.29
Normal	1	7.0		0.4		0.0	3.1			
Normal	2	7.4			0.5	0.3	1.4			
Normal	2, frozen	7.4			0.4		1.3			

* The toxicity for mice is expressed as that dilution of the rickettsial suspension used for measurements of oxygen uptake, 0.25 ml. of which will kill 50 per cent of the mice.

‡ The constancy of k_C and k_G , the ratio of the rate of oxygen uptake with casein hydrolysate and glutamate respectively to the dilution required to kill 50 per cent of the mice, is a measure of the proportionality between rickettsial viability and respiratory activity.

§ The 50 per cent immunization end-point in cotton rats for pool 2958 corresponded to a dilution of the original yolk sac pool of $10^{6.5}$, that for pool 2956 to a dilution of $10^{7.5}$, when 0.25 ml. amounts were inoculated into cotton rats.

|| These were portions of the preceding rickettsial suspensions which had been frozen and thawed.

glucose or lactate, but also no disappearance of glucose either aerobically or anaerobically, with or without addition of adenosine triphosphate, cozymase, hexosediphosphate, magnesium, and manganese. However, a very slow oxygen

uptake, roughly proportional to the rickettsial activity, occurs with pyruvate. This oxygen uptake disappears after about 4 hours, in contrast to that with glutamate, which decreases only 10 to 20 per cent in this time. The significance of the small oxygen uptake with pyruvate was checked by measurements of the disappearance of pyruvate in the presence of normal and infected yolk sac preparations. With the former, there was no change in substrate concentration (0.001 M) in 5 hours; with the latter, in one instance 1.2 micromols, with a second more active preparation, 2.3 micromols disappeared in 5 hours.

Succinate increases the oxygen uptake of rickettsiae, but in this instance there is a small oxygen uptake with similar preparations from normal yolk sac. Furthermore, there was no parallelism between rickettsial toxicity and the rate of oxygen uptake in the presence of succinate. It is possible that live rickettsiae may be impermeable to succinate since on freezing in the absence of protein or other protective substances, the viability of rickettsiae as indicated by their toxicity for mice, and their activity toward glutamate and pyruvate are greatly reduced while their rate of oxygen uptake with succinate is doubled or trebled (see table, pools 3040 and 2676).

It is clear from these results that typhus rickettsiae which have been separated from the greater part of the tissue in which they were grown exhibit definite metabolic activity. This phenomenon is quite different in nature from the changes in metabolism observed in some virus-infected tissues. The latter appear to be due to changes in the tissue metabolism brought about by the presence of the virus rather than to activity of the virus itself (1, 10).

SUMMARY

Partially purified suspensions of typhus rickettsiae have been shown to exhibit metabolic activity as evidenced by consumption of oxygen and production of carbon dioxide in the presence of glutamate. Similar activity at a much lower rate occurs in the presence of pyruvate. The rate of oxygen uptake was directly proportional to the concentration of viable rickettsiae, as estimated by their toxicity for mice. Normal yolk sac suspensions prepared in the same manner showed only a very slight oxygen uptake under the same conditions. Glucose was not metabolized by the rickettsial suspensions.

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A VIRUS RECOVERED FROM THE FECES OF "POLIOMYELITIS" PATIENTS PATHOGENIC FOR SUCKLING MICE

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PLATES 27 AND 28

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The present report describes the isolation and certain properties of a virus recovered from the feces of children having symptoms similar to those of poliomyelitis. The agent differs from poliomyelitis virus in its host range, being pathogenic for suckling mice and hamsters but not for adult mice or hamsters or for *rhesus* monkeys. The disease in the experimental animal differs from poliomyelitis in that the anatomical response is in the striated muscles rather than the central nervous system (1).

The study was undertaken in 1947 and the original plan was to test fecal suspensions in mice and hamsters from a number of outbreaks. The work was prompted by the report of Milzer and Byrd (2) that autolyzed brain suspension facilitates the isolation of poliomyelitis virus in mice, and by certain unpublished observations of the Battle Hill epidemic of poliomyelitis, which implied that such isolations might be possible. Hamsters were included because of their value in the Battle Hill work (3). After many specimens had been tested without success, it was decided to add an additional group of test animals, suckling mice. This was done because other studies in the Division had suggested that such animals are unusually susceptible, under certain circumstances, to the OT strain of mouse encephalomyelitis virus.

Nature of the Specimens

Poliomyelitis was not epidemic in up-state New York in 1947 and only 925 cases were reported to the Department of Health. Thirty (3.2 per cent) of these died. The non-paralytic cases amounted to 42.6 per cent. Twenty per cent of the patients were more than 20 years of age. The disease did not differ significantly in these respects from that in the 2 preceding years or in 1948.

The specimens were collected by district state health officers from five small outbreaks in widely separated parts of New York State. One or more fecal and blood samples were received from forty-two individuals, of whom fourteen were patients and twenty-eight were contacts.

Methods

The blood was mailed to the central laboratory in the usual fashion. The feces were brought directly to us by messenger or shipped in iced containers. When received, the feces

from the patients were stored at $-70^{\circ}\text{C}.$, those from the contacts at $-5^{\circ}\text{C}.$ This procedure has since been revised and all fecal specimens are now shipped and stored in dry ice. The sera were separated from the clots, distributed in 1 ml. amounts in small tubes, and frozen.

Preparation of Fecal Suspensions.—The feces were ground with sand in a mortar with sterile distilled water to make an approximately 20 per cent suspension. After centrifugation for 10 minutes in a horizontal centrifuge at 3000 R.P.M., ether, approximately 20 per cent by volume, was added to the supernatant fluid which was then stored in the refrigerator. The following day the ether was removed by vacuum and the suspension spun for 1 hour at 3000 R.P.M. The supernatant fluid was aspirated, plated on blood agar, and refrigerated overnight.

Specimens from patients were injected into suckling and 10 to 12 gm. mice of the Albany standard strain, and into adult hamsters. Mice (10 to 12 gm.) and adult hamsters were inoculated as routine with specimens from contacts. Suckling mice also were used for testing the specimens from the contacts of two families in which acute-phase feces from the patients were not received. Inoculations were made intracerebrally and sometimes intraperitoneally as well. The fecal suspension was injected into mice, (10 to 12 gm.), with an equal amount of autolyzed brain tissue prepared after Milzer and Byrd's directions (2). A second group received autolyzed brain tissue with sulfadiazine, since Shaw had noted an apparently more rapid onset of paralysis in mice given intestinal suspensions containing Theiler virus and streptomycin or sulfa compounds (4). The material was given with and without autolyzed tissue in suckling mice. Two adult hamsters were inoculated intracerebrally with each specimen. Animals were observed for 30 days or longer. Brain passages were made from all 10 to 12 gm. animals that appeared abnormal, and, as routine, about the 19th and 33rd day, from pooled brains of two apparently normal mice.

Neutralization Tests.—Baby mice, 2 to 8, preferably 4 or 5 days of age, were inoculated, usually intraperitoneally (0.05 ml.), with equal parts of virus dilution and serum, combined and incubated at room temperature for 1 hour. One amount of virus was used with undiluted serum in the preliminary tests. In the case of the K.H. strain, this represented, for two-thirds of the tests, approximately 100 to 200 median effective doses and between 30 and 1450 M.E.D. for all the tests included in the present report. For the T.T. strain, it represented approximately 300 to 2000 M.E.D. for three-fourths of the tests and from 85 to 2000 M.E.D. for all tests. The amount of serum or of virus was varied in certain later experiments. As a virulence control, three 10-fold dilutions of the agent were given combined with an equal amount of the diluent, physiologic salt solution containing 10 per cent infusion broth. Pooled human serum was also used as a control in each test. Where possible, the median effective dose was estimated by moving-average interpolation (5).

The use of very young mice necessitated an arbitrary evaluation of the animal tests. Mice that died during the 1st and 2nd days following inoculation and mice that were missing at any time were excluded from the calculations. Only those dead, moribund, or paralyzed during the critical period of the test were counted. The test period was usually 12 to 14 days and the mice were sacrificed when paralyzed, except in a few tests in which they were followed to learn whether improvement would occur.

The neutralization tests with one dose of virus were interpreted as follows. When the survivors were fewer than 30 per cent of the test group, the result was considered negative. When the survivors amounted to 30 to 45 per cent, a trace of activity was indicated. Forty-five to sixty per cent survival was given a value of \pm to denote moderate neutralization, and more than 60 per cent survival was considered to show that definite neutralization had occurred (+).

Neutralization tests using the Lansing strain of poliomyelitis virus were observed for 21 days and 1st day deaths disregarded.

Immune Sera.—Immune sera were prepared with the T.T. strain in large mice and adult hamsters. Mouse or hamster brain suspension (10 per cent) containing the formalized or living agent was injected intraperitoneally. The mice received increasing amounts from 0.1 to 0.5 ml. in three weekly series of three daily doses. The hamsters were given four weekly doses of 1.0 ml.

Bleedings were taken from 7 to 13 days after the last injection. The serum, without preservative, was stored in the frozen state.

Histologic examination of selected animals has been practiced throughout. The specimens, in the case of suckling mice the entire animal, were fixed in Zenker's fluid plus 5 per cent glacial acetic acid and sectioned at several levels. The preparations were stained with hematoxylin and eosin and with Giemsa's solution.

Results of the Animal Tests

Acute-phase fecal specimens were available from ten of the fourteen patients and were tested by all four methods; that is, with autolyzed brain, with autolyzed brain plus sulfadiazine, in suckling mice, and in adult hamsters. Two samples, T.T. and K.H., yielded a transmissible agent in suckling mice. All the other tests failed.

Specimens taken during the acute phase of the patient's illness from sixteen of the contacts were tested in 10 to 12 gm. mice, using autolyzed brain tissue suspensions with and without sulfadiazine. All sixteen failed to yield an infectious agent, as did the four that were also tested in suckling mice and adult hamsters. In one instance, weakness of the extremities was noted in suckling mice inoculated with a suspension of feces from a contact and in one or more mice of the third, fourth, and fifth subsequent transfers. The inoculum was found to contain Gram-positive cocci, and a bacteria-free filtrate did not induce paralysis. A second test from the original fecal specimen was also negative.

The two fecal specimens from which an agent was isolated had been collected 5 and 12 days after the onset of symptoms. Of the eight specimens from patients that failed to infect suckling mice, three had been collected on the 5th day, one on the 7th, one on the 10th, and one on the 12th day. The date of collection of the other two was not determined, but they were received on the 5th and 14th days. Thus there was no relation between the time of collection of the specimens and the success or failure in isolating an agent. In several cases, the feces had been held at refrigerator temperature for some days before being sent to the laboratory.

Table I summarizes all the 1947 animal tests, including those made of the two positive cases and their contacts. It will be noted that three separate isolations were made from the acute-phase fecal specimen of K.H.; and from T.T. two isolations were made from one acute-phase specimen and one isolation from a second acute-phase specimen. The suckling mice were paralyzed in the case of T.T. on the 9th, 6th, and 5th days; in the case of K.H., on the 7th, 9th, and 11th days. Since eight other specimens did not induce paraly-

TABLE I
Animal Tests of Patients' and Contacts' Feces Collected in 1947

Place	Status	Age	Mice	Suckling mice	Adult hamsters
Cortland	Patient	yrs. 5	0	0	0
	Contact	2	—	—	—
	"	3	—	—	—
	"	30	—	—	—
Jamestown	Patient	4	0	0	0
	"	8	—	—	—
	"	9	—	—	—
Ithaca	Patient	6	—	—	—
	"	7½	—	—	—
	"	10	—	—	—
	"	23	0	0	0
	"	34	—	—	—
Binghamton	Patient	6	0	0	0
	"	10	—	—	—
	"	32	—	—	—
	Contact	3½	—	—	—
Coxsackie	Patient	9	—	+	—
				+	
				+	
	Contact	2	—		
	"	11	—		
	"	15	—		
	"	32	—		
	"	37	0		
	"	66	—		
	Patient	3½	—	+	—
				+	
				+	
	Contact	6	—		
	"	6½	—		
	"	12	—		
	"	31	—		
	"	37	—		
	"	38	—		
	"	41	—		

+ indicates that the agent pathogenic for suckling mice was isolated.

— indicates virus not isolated.

0 indicates not tested.

sis and these two did in each of six trials, we may assume that the agent was present in the patients' feces and not in the test animals.

Twenty-six serial transfers of normal suckling mouse brain suspensions at 4 to 5 day intervals have been made without evidence that our colony harbors a latent infection.

Inoculation of Monkeys.—Young *rhesus* monkeys (*Macacusc mulatta*) were inoculated with the two fecal specimens that yielded the agent, and also with three mouse brain suspensions from subsequent generations.

The suspensions were prepared as before except that ether was not added to the fecal suspensions given intranasally. The monkeys were inoculated intracerebrally (0.2 ml.), intraperitoneally (0.3 and 0.5 ml.), and intranasally (0.5 ml.). Three were given 0.5 ml. intranasally on 4 successive days. Some of the intraperitoneal inoculum was infiltrated into the abdominal muscles. Temperatures were taken daily.

Monkey 4-24 was inoculated with a 10 per cent suspension of T.T. feces. No fever or other signs of illness were noted. The same suspension paralyzed nine of ten suckling mice between the 5th and 8th days.

Monkey 4-20 was inoculated with a 10 per cent suspension of the feces of K.H. No response was seen. The same suspension caused paralysis of seven of twelve suckling mice between the 7th and 11th days.

The tenth mouse generation of the K.H. strain and the second and sixteenth generations of the T.T. strain were similarly tested in monkeys. The second generation preparation, which had been stored in a dry-ice box for 2 months, was inactive in mice and one monkey. The others caused prompt paralysis of suckling mice but no response on the part of the monkeys.

Thus, four preparations infectious for suckling mice, including both of the acute-phase fecal specimens, failed to induce signs of disease in *rhesus* monkeys. While the tests are not numerous, they suggest that monkey-pathogenic poliomyelitis virus was not present in the patients' feces and that the agent recovered from the feces is not pathogenic for monkeys. Sera collected from two of these monkeys 1 month following inoculation failed to neutralize the agent.

Two attempts have so far been made to infect newborn guinea pigs. Three guinea pigs approximately 5 hours old were inoculated by the intracerebral route with a mouse brain suspension of the K.H. strain highly infectious for mice. No signs of disease were detected. Six subsequent intraperitoneal injections failed to produce paralysis or to stimulate the production of antibodies. Three guinea pigs less than 1 day old appeared normal after intracerebral inoculation of a mouse-virulent suspension of T.T. Their sera were not tested.

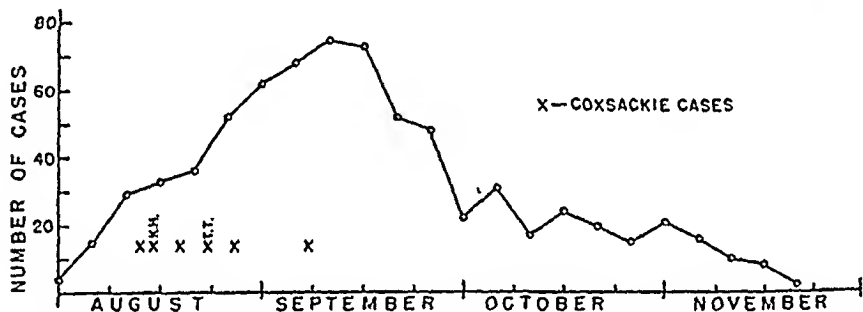
Nature of the Clinical Disease

The original isolations were made from two children who lived in a Hudson River Valley village (population, 2300). Six similar illnesses which occurred within the village during August and early September, 1947, were diagnosed as poliomyelitis. Their occurrence coincided with the rising incidence of polio-

myelitis throughout the state (Fig. 1). All the patients were children and three were paralyzed.

T.T. was a 9 year old boy who complained of headache, nausea, and pain in his legs on Aug. 22. The following day he was febrile (104.0°F.); there was appreciable weakness in both legs, but no nuchal rigidity. His trunk and back muscles became weak and he was hospitalized. His cerebrospinal fluid was slightly cloudy, colorless, contained 250 red blood cells and 64 leucocytes per c. mm., 50 mg./100 ml. of sugar, and slightly increased globulin. During his hospital stay, the child required catheterization once. Weakness of the back muscles was a prominent finding and this persisted throughout the fall and winter. The patient was transferred to the New York State Reconstruction Home. Seven months later he was still unable to raise himself from a recumbent position. A year later he was able to discard his back brace except while playing out of doors and was no longer aware of any disability.

K.H. was a $3\frac{1}{2}$ year old boy whose illness began on Aug. 14 with sore throat and lethargy. Two days later the adductor muscles of his left thigh were found to be very weak. The



TEXT-FIG. 1. Reported cases of poliomyelitis in New York State, exclusive of New York City, 1947.

history does not mention nuchal rigidity. His cerebrospinal fluid contained 10 red blood cells, 2 polymorphonuclear and 2 mononuclear leucocytes. Globulin was not increased. The sugar was estimated to be 50 mg./100 ml. The patient was later transferred to the New York State Reconstruction Home with weakness of the adductors of the left thigh and inversion of the left foot on walking. Paralysis was not recognizable 8 months later.

These two patients lived within a short distance of one another but did not become acquainted until they were hospitalized. The third child known to have been severely paralyzed lived on the outskirts of the village.

The sanitation in the village is satisfactory. The area is one in which poultry and dairy farming form the major enterprises. One large poultry farm is near the home of T.T. The children had not left the village and no record of exposure to a known case of poliomyelitis could be secured. The local physicians recalled other children who had minor complaints during the summer, including headache, nausea, fever, and leg pains but they recovered rapidly and without sequelae.

Relationship of the Agent to the Patients

The recovery of a virus does not constitute proof that it has been responsible for the patient's disease. It is usually necessary that an immune response to the agent be demonstrable. Accordingly, neutralization tests have been per-

formed using the acute- and convalescent-phase sera from the patients and the agents recovered from their feces.

The results of a representative experiment are summarized in Table II. It is evident that the acute-phase serum from K.H. had no neutralizing activity for either strain under the conditions of the test, while the specimen collected 24 days later neutralized both. The acute-phase sample from T.T. had neutralizing activity which increased tenfold within 23 days and diminished in the following months. The median effective dose of acute-phase serum against approximately 125 M.E.D. of T.T. virus was 1:26. Twenty-three days later it had risen to 1:260 and 8 months later had fallen to 1:10. It can be assumed, therefore, that both patients were infected with the agents isolated from their feces at the time that they were ill.

Inhibitory Effect of Feces Collected during Convalescence

Convalescent fecal specimens of T.T. and K.H. collected 28 and 35 days, respectively, after onset of symptoms were also tested for antiviral activity. This was undertaken because other work in this laboratory has shown an inhibitory principle in feces of mice infected with mouse encephalomyelitis virus (6) and the report of similar activity in the feces of monkeys convalescent from poliomyelitis (7).

The fecal suspensions were prepared as before with omission of the treatment with ether. One portion of 20 per cent suspension was filtered through a Mandler candle and a second portion was treated with streptomycin (100,000 units per ml. of suspension) and sodium penicillin (4,000 units per ml.). Both preparations were tested by mixing equal parts of fecal suspension and mouse brain virus preparations and incubating for 3 hours at room temperature before inoculating suckling mice. The test animals were injected intraperitoneally. An unrelated fecal suspension previously found to be non-infectious for suckling mice was used as control.

The results are summarized in Table III. They suggest that the late specimen from T.T. had a measurable antiviral effect.

Nature of the Agent

The agent has so far been tested in mice, hamsters, guinea pigs, monkeys, and fertile hens' eggs, and of these only the first two have shown signs of disease. Eighty-four unsuccessful attempts have been made to induce paralysis in 10 to 12 gm. mice of the Albany standard strain by intracerebral inoculation of virus from the first to the fortieth generation. The 12th day of life is apparently near the end of the period of mouse susceptibility. Mice 11 and 12 days old became paralyzed but the incubation period was somewhat longer than usual. Among three families of mice 14, 15, and 16 days of age, comprising twenty-seven animals, there was only one mouse with doubtful paralysis (15 day old, 7th day).

TABLE II

Immunologic Response of Patients T.T. and K.H. to the T.T. Strain of Virus

Serum	Date of bleeding	Dilution	Virus suspension dilutions			
			10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴
Broth salt			-----	4,4,4,4,4,4,4	4,4,4,5,5,5, 5,6,6,8	4,6,8,S,S,S, S
Normal human serum pool		Undiluted	S,S,S,S,S,S, S,S,S,S,S			
" "		1:5	4,5,5,5,11, S,S,S			
" "		1:50	3,3,3,3,3,3,3			
K.H.	8/26/47	Undiluted	3,3,3,3,4,4,4			
"	9/19/47	Undiluted	S,S,S,S,S,S,S			
"	"	1:5	5,6,S,S,S,S, S,S,S			
"	"	1:50	4*,4*,4*,4, 4,4,4,5,S			
T.T.	8/26/47	Undiluted	5,5,S,S,S,S, S,S,S,S			
"	"	1:5	8,S,S,S,S,S, S,S			
"	"	1:50	4*,4,4,5,5,5			
"	9/18/47	Undiluted	S,S,S,S,S,S, S,S,S			
"	"	1:5	1,2,S,S,S,S, S,S,S,S			
"	"	1:50	7,S,S,S,S,S, S,S			
"	5/14/48	Undiluted	S,S,S,S,S			
"	"	1:5	6*,6*,S,S,S, S,S			
"	"	1:50	4*,4,4,4,4, 5,5,5,5			

Survivors are shown by "S." The numbers indicate the day on which the animal was found paralyzed, dead, or missing. Paralyzed animals are indicated by bold-faced numbers. Asterisks signify missing mice.

The agent is stable in mouse brain suspension at -70°C . for at least several months and the fecal specimens were infectious $9\frac{1}{2}$ months after collection. It

TABLE III
Tests of Convalescent Patients' Feces for Virus Inhibition

Patient	Dilutions of K.H. Virus			Dilutions of T.T. virus		
	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}
Broth salt				3, 3, 3, 3* 3*, 3*, 3*	3, 3, 3, 3, 3	4, 5, 5, S, S
Broth-salt + antibiotics	4, 4, 4, 5, 5, 5, 5	5, 5, 5, 6, 9	4, 5, 6, 6, S S, S, S, S	3, 3, 3, 3, 3, 3, 3*	3, 3, 3*, 3*, 4, 4, 4, 4, 4, 4, 4*	3*, 3*, 3*, 3*, 4, 4, 4*, 5*, S, S
T.T. + antibiotics	5, 8, 12, S, S, S, S, S, S, S, S	4, S, S, S, S, S		5, 5, 5, 5, S	9, S, S, S, S, S, S	
T.T., filtered				4, 4, 4, 5, 5*, S, S, S, S	S, S, S, S, S, S, S, S	
K.H. + antibiotics	3, 3, 3, 4, 4, 4*	3, 3, 3, 3, 4, S, S		3*, 3*, 3*, 3*, 3*, 3	3, 3, 3, 3, 3, 3, 3, 3, 3	
K.H., filtered				3, 3, 3, 3*, 3*, 3*	3, 3, 3, 3, 3*, 3*	
K.C. + antibiotics (control)	3, 3, 3, 3, 3, 3	4, 4, 5, 5, 5, 5, S	-			

The convalescent patients' fecal specimens were collected 28 days (T.T.) and 35 days (K.H.) after the onset of disease.

Survivors are shown by "S." The numbers indicate the day on which the animal was found paralyzed, dead, or missing. Paralyzed animals are indicated by bold-faced type. Asterisks signify missing mice.

is also stable in 50 per cent glycerol for at least 5 months. It is inactivated by 0.25 per cent formalin at room temperature.

Cultural Tests.—Infected mouse brain suspension, K. H. and T.T., filtered through Mandler candles, induced paralysis in suckling mice and hamsters but failed to initiate growth at 35°C . in casein hydrolysate semisolid agar containing sodium thioglycollate. No significant growth was obtained aerobically from unfiltered infected brain suspension on beef extract agar, horse blood agar, or potato infusion-sheep blood agar plates.

Microscopic and cultural examination of unfiltered mouse brain suspension infected with the T.T. strain failed to detect the presence of microorganisms of the pleuropneumonia group or of any bacteria. The media used were beef heart infusion broth containing 30 per cent ascitic fluid and broth with 30 per cent normal horse serum. Beef heart infusion agar with 30 per cent ascitic fluid and agar containing 30 per cent normal horse serum were also included. Seven serial transfers were made in each fluid medium at 2 to 4 day intervals; 0.2 ml. amounts were tested on the surface of the solid media in plates at the same time. All ascitic fluid and serum agar plates were incubated for 7 days or longer. Darkfield and Gram-stained preparations were made of the original material and several of the fluid media.

TABLE IV
Neutralizing Activity of Sera of Patients and Household Contacts

Individual	Status	Age	Test virus					
			T.T.		K.H.		Lansing	
			Acute	Conva-lescent	Acute	Conva-lescent	Acute	Conva-lescent
K.H.	Patient	3½	—	+	—	+	?	?
K.C.	Cousin	6			—	—	+	+
B.C.	"	12			—		+	
R.H.	Mother	31			+	+	+	+
B.C.	Aunt	37			—	±	+	+
K.C.	Uncle	38			+	+	+	+
C.H.	Father	40			—	—	+	±
T.T.	Patient	9	+	+	+	+	?	?
E.T.	Brother	11	—	—			+	+
W.T.	"	15	+	+			+	+
G.T.	Mother	32	+	±			+	+
N.T.	Father	37	—	—			+	+
R.T.	Grand-mother	66	—	—			+	+

Size.—Preliminary tests indicate that the virus is small. A 10 per cent suspension of mouse brains infected with the K.H. strain was centrifuged for 30 minutes in an air-driven Beams and Pickels type ultracentrifuge at 350, 450, and 550 r.p.s., approximately 50,000, 75,000, and 100,000 times gravity. The undiluted supernatant fluids from all three runs proved to be virulent for suckling mice. A 10 per cent suspension of mouse brains infected with the T.T. strain, which has a higher titer than the K.H. strain, was ultracentrifuged at 550 r.p.s. for 30 minutes. The supernatant fluid, diluted to 10^{-2} , was infective for 100 per cent of the mice. Greater dilutions were not made in this experiment. Preliminary measurements based on Elford membranes and examination with the electron microscope confirm these results and suggest that the virus is less than 40 $m\mu$ in diameter and approximately spherical.

Tests of the Sera of Household Contacts

The sera of the patients' household contacts have been tested for the presence of neutralizing activity for the T.T. and K.H. strains and also for the Lansing

strain of poliomyelitis virus. The results are summarized in Table IV. It will be seen that of the ten from whom convalescent-phase serum samples had been secured, five had neutralizing activity for the new agent. All the samples neutralized the Lansing virus. The sera of all but the very young in New York usually neutralize Lansing virus. The different behavior of these sera with the new agent indicates either that the maturation effect which has been postulated as an explanation of the reaction of adult sera with poliomyelitis virus (8) does not apply, or that infection with the new virus is not prevalent or has not occurred frequently in the past. The persistent or repeated prevalence of virus is an alternative explanation of the ubiquitousness of antipoliomyelitis serum activity. Taken at face value, the tests suggest that one brother and the mother of T.T. had been infected, and also that three adult members of the household of K.H. had been infected with the new agent.

Sera from other poliomyelitis patients in New York have also been tested by the same methods. Neutralizing activity was found in acute- and convalescent-phase sera of two of nine patients, both adults. Twelve pairs of sera have been received from Dr. Thomas Francis, Jr. They were collected during the summer of 1947 from Michigan children with indefinite symptoms. Both sera of two patients reacted strongly with the K.H. strain and two others gave weak reactions.

Nature of the Disease in Mice

The incubation period of the T.T. and K.H. strains is usually 3 days. After the ninth generation, approximately 70 per cent of passage mice inoculated intracerebrally with brain suspension 10^{-1} showed paralysis or other signs of infection on the 3rd day. A few were paralyzed on the 2nd day and approximately 17 per cent on the 4th day. The small number of animals showing signs of disease on the 5th, 6th, 7th, 8th, and 9th days were mainly from families 9, 11, and 12 days old.

The prodromal signs of disease in the baby mice are lethargy and generalized weakness of the body and extremities, delayed response to touch, a tendency to move in circles suggestive of unilateral leg weakness, and at times stunted growth and poor nourishment. Paralysis or death follows by a day. One or more extremities may be paralyzed. The legs may remain flexed or extended, while the toes fail to spread and pressure on them produces no reaction. Some mice develop labored breathing. Spasms and convulsions have not been observed. In the few mice that have lived for more than the day following the prodromal signs, the paralysis has become progressively more severe and generalized. Some have developed swelling and induration of the muscles and others have shown apparent swelling of the joints. Such mice are conspicuously stunted, probably in part because they are unable to suckle.

Mice that have been paralyzed for a short period show no gross lesions. Animals paralyzed for a day or more show opaque, whitish muscles, especially in the pectoral group, the *longissimus dorsi*, and in paralyzed extremities. Severely paralyzed muscles are firm to

touch and very white. Gross lesions have not been observed in the viscera or organs of the central nervous system.

Twenty-five suckling mice and two suckling hamsters have been extensively examined histologically. None of the animals inoculated with either strain has had lesions of the central nervous system but nearly all have shown widespread changes in the skeletal muscles. The lesions begin as a hyaline degeneration of the muscle fibers, followed by complete destruction. The fibers are transformed into amorphous masses, the fragments being quickly absorbed and phagocytosed (Figs. 1 to 3). Regeneration is evident from the first, and large masses of young, actively multiplying muscle cells give the lesion a very cellular appearance. In one mouse, examined 7 days after the initial symptoms, repair was so extreme that the lesion resembled a rhabdomyosarcoma (Figs. 4 to 6). The lesion resembles Zenker's degeneration as it occurs in a variety of apparently unrelated conditions. We have observed similar lesions in the spinal muscles in hamsters infected with MM virus and in mice infected with other neurotropic agents. A comparison of these lesions is being made at present. Study of the terminal nerve structures is incomplete. Little has been found in the other organs. Myocarditis, said to occur in EMC virus infection (9, 10) and which we have once observed, as well, in a mouse infected with mouse encephalomyelitis (OT) virus, has not been found.

Further Isolations of the Agent

Agents that induce similar signs and lesions in suckling mice have been isolated from other outbreaks and isolated cases of apparent poliomyelitis. Nine isolations were made during 1948 from patients ill during that summer in New York. Only one had been frankly paralyzed although another, an adult woman, had questionable weakness of the leg. Three of the strains differ from the T.T. and K.H. strains. They induce lesions of the central nervous system as well as less severe changes in the muscles. They may be distinguished by the behavior of infected mice.

Five isolations have been made from fecal suspensions of patients from the 1947 Wilmington, Delaware, epidemic. The material was supplied by Dr. Robert Ward, New York University, College of Medicine, and by the Communicable Disease Center, United States Public Health Service. Three of these patients were described as having had weakness of certain muscles. Since numerous attempts by others to recover monkey-paralyzing virus from the Wilmington outbreak had failed, and because the disease was unusual epidemiologically and clinically (11, 12), a comprehensive study of the materials at hand is being made.

It seems evident that the agent is at present disseminated rather widely, whatever its importance may be as a cause of disease. The newer strains reveal apparent antigenic differences and also other variations that are still being investigated.

Relationship to Other Viruses

Other viruses that induce similar lesions in the striated muscles—mouse encephalomyelitis, Columbia SK, MM, and EMC viruses—do not selectively paralyze suckling mice and do induce lesions of the central nervous system.

Columbia SK, MM, and EMC viruses are serologically related (13, 14). Serum produced with one of them, MM virus, did not neutralize the new agent nor did rabbit antiserum or mouse antiserum of FA mouse encephalomyelitis virus.

Immune animal sera for Newcastle disease, lymphocytic choriomeningitis, and the Aycock and Lansing strains of poliomyelitis have also failed to neutralize the agent. Adult normal mouse and normal rabbit sera were inactive under the conditions of the test but the serum of one normal monkey of the several tested had a slight neutralizing effect.

DISCUSSION

The isolations to date have all been from patients diagnosed as having had abortive or paralytic poliomyelitis, but, since no effort has yet been made to recover virus from patients with other diseases or from healthy individuals, no conclusions based on the association seem warranted. Sufficient specimens have been examined to indicate that carriage of the agent is not commonplace.

The deficiencies in our information that have seemed most important to us are due to the lack of human material. It would obviously be most valuable to be able to examine the tissues of an individual infected with the agent. Indeed it has seemed to be premature to suggest a name for the virus or the disease with which it has been associated in the absence of knowledge of the kind of anatomic response it induces. Similarly, recovery of virus from human organs rather than from feces would be a matter of importance, especially if the presence of the virus and a lesion were associated. No such specimens have been available. We have examined a limited number of specimens of nervous tissue from recent fatal cases of poliomyelitis without success, but in none of these had the suckling mouse virus been demonstrated in the feces. Muscle biopsies would obviously be valuable specimens both as material for histologic examination and for animal tests. Fifty-four specimens of spinal fluid have been tested in suckling mice with negative results.

It should perhaps be pointed out that, while we have not found lesions in the brains or spinal cords of our mice, we regularly use these tissues for the transfer of virus since the infectivity titer is high and the brain is a uniform and easily harvested sample.

The muscle lesions in the immature mice may be similar to those described in monkey and human poliomyelitis by Carey and his colleagues (15, 16) and in man by Dublin, Bede, and Brown (17). These authors paid particular attention to the terminal nerve structures but Carey *et al.* described degeneration of the muscle fibers, including loss of striations and hyalin changes, and Dublin reported atrophy of the muscle fibers ending in dark, pyknotic masses. The material examined by Dublin and his associates was from children 35 to 48 days following the onset of poliomyelitis. Carey had examined earlier stages of the

process and found changes in the myoneural junction within 36 hours of infection.

Muscle fiber degeneration of the kind seen in our suckling mice occurs to a limited extent during MM virus infection in the hamster. Similar lesions were found by Rustigian and Pappenheimer (18) in the muscles, at the site of injection of mouse encephalomyelitis viruses and SK (Columbia) virus. They distinguished between these lesions and the inflammatory infiltrate that follows the intramuscular injection of lymphocytic choriomeningitis virus and the non-specific reaction in muscles to certain other neurotropic virus inocula. The last, they believe, is a reaction to brain tissue rather than to virus. Thus, the presence of myositis of a striking kind has recently assumed considerable significance in the pathogenesis of poliomyelitis and poliomyelitis-like diseases and has, in certain instances, been found to resemble the lesions that occur in suckling mice and hamsters.

It may be well to mention other similarities between poliomyelitis and the disease under consideration. The clinical similarities have been mentioned. They cannot be satisfactorily analyzed on the evidence we have so far accumulated. The seasonal occurrence of both diseases appears to be the same. Both viruses are unusually small and relatively stable in glycerol. Both occur in the feces.

These similarities are listed not with the thought of suggesting that the two diseases, or viruses, are necessarily closely related but to call attention to the possible difficulties in studying outbreaks of poliomyelitis if such a disease as the present one occurs simultaneously. This may have occurred during the past two summers in New York and very probably did in Delaware in 1947, since Melnick isolated monkey-pathogenic strains of poliomyelitis virus from patients in the suburbs of Wilmington (19) while we recovered the new agent from urban patients. One is reminded of the 1934 epidemic of poliomyelitis in Los Angeles (20-22). It occurred earlier in the summer than is the rule, the number of patients more than 10 years of age was abnormally high, the mortality rate exceptionally low, and there was striking evidence of high communicability. Multiple cases occurred in 12.5 per cent of the households that were afflicted. Poliomyelitis virus was recovered from a number of the fatal cases (23) and from the nasal washings of one mild case (24). It is possible that classical poliomyelitis occurred simultaneously with an outbreak of a second but more benign disease. Under such circumstances the isolation from fatal cases of virus pathogenic for monkeys would be of limited significance since the sample would be drawn from the more virulent infection, while the unknown disease, by its numbers, would account for the peculiarities of the epidemic. Something of this kind may well have occurred in Wilmington and an effort should be made to test as many specimens as possible to determine whether it did happen.

SUMMARY

A virus has been recovered from the feces of two children having symptoms similar to those of poliomyelitis. The virus is pathogenic for suckling mice and hamsters but not for *rhesus* monkeys. It induces striking lesions in the skeletal muscles of the experimental animal but not in the central nervous system. Other viruses inducing similar signs and lesions in suckling mice have been isolated from several other outbreaks of a poliomyelitis-like disease, including one large urban epidemic.

The examinations for microorganisms of the pleuropneumonia group and the electron microscope observations were made by Miss Julia M. Coffey; the ultrafiltration and ultracentrifugation studies, by Mr. James J. Quigley. Infectivity tests in embryonated eggs were made by Dr. Irving Gordon.

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EXPLANATION OF PLATES

PLATE 27

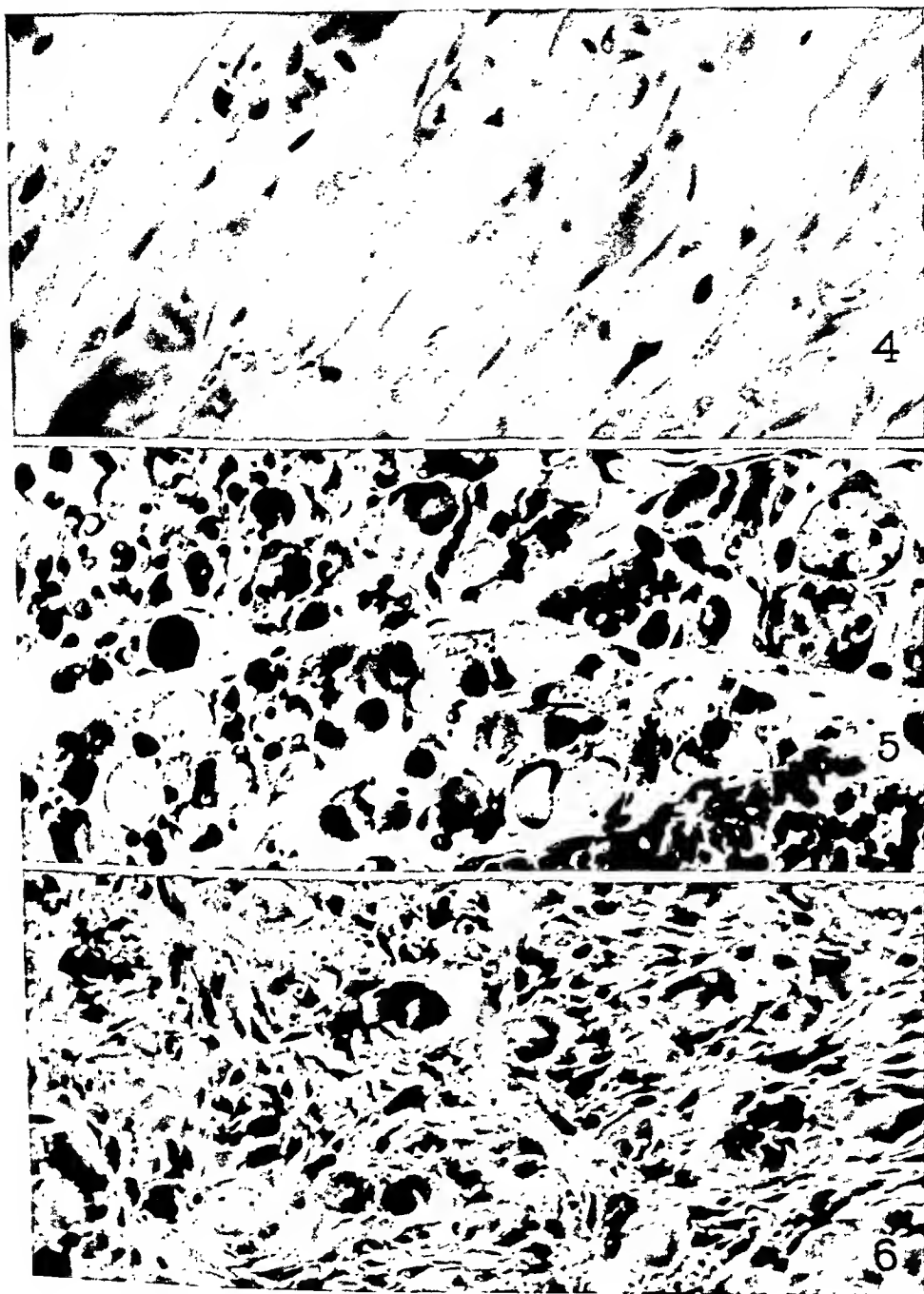
FIGS. 1 to 3. The usual appearance of the muscles in paralyzed suckling mice. Fig. 1 shows muscle of a leg; Fig. 2, the muscle of a thoracic wall and Fig. 3, that of the abdominal wall. $\times 140$.



(Dalldorf *et al.*: Virus from feces of "poliomyelitis" patients)

PLATE 28

FIGS. 4 to 6. The stages in the evolution of the lesion in striated muscle. Fig. 4 represents the early phase in which degeneration of adult muscle fibers is the dominant lesion. In Fig. 5 the process is well advanced and phagocytosis of deteriorated fibers and regeneration of muscle cells are prominent. Fig. 6 illustrates the lesions seen in a mouse which survived 7 days. Note the extreme cellularity of the repair. $\times 580$.



(Dalldorf *et al.*: Virus from feces of "poliomyelitis" patients)

PERIPHERAL VASCULAR REACTIONS IN ANAPHYLAXIS OF THE MOUSE

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PLATE 29

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In the course of studies on the site of antibody formation it was found that mice sensitized to horse serum showed extraordinary vascular reactions in the ears when reinjected with the same serum at an appropriate interval. The phenomena, observed under the microscope, appeared not only in animals showing anaphylactic shock but also in many which presented no other apparent signs of anaphylaxis. That is to say the vascular changes in the ear seemed to constitute a sign of anaphylactic sensitivity far more delicate than the production of anaphylactic shock itself. It seemed likely that these reactions could be used for the study of the mechanisms of local hypersensitivity. Further, it seemed probable that by means of these reactions the mouse could be used as a convenient laboratory test animal to take the place of larger or more costly animals for various immunological studies.

Because of erroneous statements in the older literature (1-7) it is still believed, even by many immunologists and allergists, that the mouse is not susceptible to anaphylactic shock, and the possibility of using the vascular changes in its ears for immunological research has, of course, not been considered. Under these circumstances it seemed wise to make a study of the characteristics of the peripheral vascular responses in mice showing anaphylactic shock in all degrees of intensity, not only to learn more about them but to determine whether they are a true part of the anaphylactic reaction—and hence an indication of the animal's sensitivity—or whether they are merely secondary to blood pressure changes or nerve stimuli.

The present paper describes the findings of such a study carried out in mice sensitized to various sera and reinjected later with the same material. The vascular changes will be fully described since they have brought out some new findings of physiological interest quite apart from their immunological implications.

Although a number of workers (8-17) have observed anaphylactic shock in the mouse their studies have not been aimed at an understanding of the circulatory changes taking place in this animal. On the contrary little is known about this matter. Fortunately for us methods for observing the most minute as well as the larger blood vessels in the ears (18) or claws (19) of mice, and for measuring their blood pressure during and after anaphylactic shock were already at hand in this laboratory (19).

Previous Work.—In spite of the fact that several authors (1-7) reported between 1908 and 1910 that mice are refractory to anaphylactic shock, papers by Braun (8, 9) and Schultz and Jordan (10) appeared within about a year of each other, proving the contrary. Shortly thereafter Ritz (11) and von Sarnowski (12) also produced anaphylactic shock in mice, and in 1926 Schiemann and Meyer (13) obtained both active and passive anaphylaxis. In 1937 Bourdon (14) reported active sensitization of white mice and recently Weiser, Golub, and Hamre (15) have restudied the subject and discussed the previous findings. The mouse has also been used by Mayer and Brousseau (16) and also by Perry and Darsic (17) to gage the activity of antihistaminic drugs and to study histamine shock.

The Induction of Anaphylactic Shock in Mice

In the present experiments trial and error showed that mice could be rendered anaphylactically sensitive, as a rule, by injecting 0.03 cc. of horse, pig, or rabbit serum into the peritoneal cavity twice, at an interval of 48 hours. After an appropriate period shock was induced by injecting into a tail vein 0.05 to 0.15 cc. (usually 0.1 cc.) of the sensitizing serum, per 30 gm. of body weight. Optimum results were obtained when the mice were shocked 16 to 35 days after the first sensitizing injection. Under these circumstances about three-fourths of the mice showed the pronounced shock symptoms that will be detailed below. About 20 per cent of them died 20 minutes to 2½ hours after the injection, and another 10 to 20 per cent died during the next 12 hours. The reactions were less regular and less severe when shorter or longer time intervals elapsed between the sensitizing and shocking injections, but shock was obtained as early as the 9th day and as late as 6 months after the first sensitization. Longer intervals have not been tried. Usually, however, shocking injections made at an interval of more than 7 weeks gave irregular findings.

Types of Experiments

Normal white mice of the Rockefeller Institute strain were employed throughout. Both unanesthetized and anesthetized animals were shocked in order to correlate the time relationship between the physiological changes, which could be best observed in the latter, and the behavior phenomena displayed by the former. Single intraperitoneal injections of pentobarbital, 0.5 cc. of a 1 per cent solution per 30 gm. of body weight, yielded suitable anesthesia with which to observe the circulatory changes in the ears or claws and to measure the blood pressure changes in the carotid and femoral arteries. These observations were made before, during, and after shock, by methods to be outlined below. The blood pressure measurements could, of course, be carried out only in the anesthetized animals, but it should be stressed here that the vascular changes, observed to the best advantage in the anesthetized mice as described further on, were also observed, with some difficulty, taking place in unanesthetized ones, at the same times after the injection of antigen and with the same intensity as in the anesthetized animals. It follows that anesthesia was not responsible for the findings.

Signs of Anaphylactic Shock in Unanesthetized Mice

The earlier workers are agreed (8-17) that anaphylactic shock in the mouse is a less stormy event than in the guinea pig or rabbit. For the first 5 or 10 minutes unanesthetized mice show merely agitation or hyperexcitability (15). Later they scratch themselves (10), the hair becomes ruffled, respiration is difficult, and some throw themselves here and there (12) or have brief convulsions with long, quiet periods between (15). Cyanosis in the ears and feet has been noticed (12, 15), but a prior blanching has not been mentioned although, as reported below, it appears regularly soon after the shocking injection is given. There is often a frog-like posture with the hind legs extended behind the body (15). Death does not come on

rapidly as in shocked guinea pigs and rabbits, but after 15 to 60 minutes (15), or it may occur after many hours.

We have found the picture of shock induced by horse, pig, or rabbit serum in unanesthetized mice substantially like that described above, with one great exception. Many of the animals showed either no signs when observed in the gross, or at most only agitation and restlessness; but when their ears were examined at low magnification after they had been placed in a holder, one observed striking vascular reactions like those now to be described as occurring in perfectly quiet anesthetized mice. They differed only in degree, being less severe, than those seen in mice with outspoken anaphylactic shock.

PERIPHERAL CIRCULATORY CHANGES TAKING PLACE IN ANESTHETIZED MICE WITH ANAPHYLAXIS

In contrast to the delay in the appearance of the manifestations of anaphylaxis, the microscope revealed profound physiological changes in the blood vessels of the ears and claws taking place promptly. They appeared even during the injection of antigen, and long before unanesthetized animals showed any signs of distress.

Changes in the Circulation of the Ears.—In more than 300 experiments anesthetized normal or sensitized mice, approximating 30 gm. in weight, were placed prone in plastaline moulds with their ears spread out upon white porcelain plaques, in a manner previously described (18-20). By these means even the most minute, as well as the larger, vessels were observed in the intact ears under the microscope. Next, one worker injected 0.05 to 0.15 cc. of serum into a tail vein while another observed the ear vessels during the injection and for various periods thereafter. The injection required 30 seconds to 1 minute.

At any time from 28 seconds to 7 minutes after beginning the injection, but usually between the 50th and 90th seconds, vascular reactions began, if the animals were sensitive.

Changes in the Arteries.—Usually there appeared first one or more brief, partial contractions extending all along the arteries of the mid and peripheral portions of the ears. In a second or two the vessels returned to their initial calibers. Momentarily the circulation increased in speed, but then, after a few seconds, it became much slower than it had been before the injection. Next, sharply localized contractions appeared in many arteries. Some promptly relaxed, but others persisted. In highly sensitive animals the localized spasms increased in number and intensity, until, within a minute or two, all arterial vessels were completely constricted and had disappeared from view. In less sensitive mice only some of the arteries showed a complete obliterative spasm while in the others the local constrictions remained, trapping blood in the vascular segments lying between them. In poorly sensitized mice only a few localized spasms were seen. More will be said of these differences below.

In those instances in which vascular spasm did not occur for a minute or two, the slowing of the circulation was the most prominent feature. Cells, moving in clumps, separated by plasma could be seen, as though they had become sticky and adherent. In rare instances flow ceased in ear arteries which were still patent, showing that spasm must have occurred in larger, more centrally situated arteries than those of the ear. In the vessels that had become completely occluded blood flow stopped of course, but in the vessels in which constriction was partial the flow merely remained slow.

Changes in the Veins.—The veins also contracted. Usually sharply localized, ring-like constrictions occurred, obliterating the lumina of the vessels for only a small fraction of a

millimeter while the greater part of the veins remained widely open. As in the arteries, many of the constricted portions relaxed after a few seconds while new spasms appeared elsewhere, or, the original constrictions remained while the newer ones occurred, until the veins became segmented in appearance, or completely constricted. Occasionally widespread constriction of entire vessels took place at once.

The intensity of the reactions varied much from one batch of mice to another. By and large about 20 per cent of the sensitized animals showed almost complete obliterative spasm of all the ear arteries and veins for several minutes. In another 30 per cent spasm of the vessels was severe enough to produce complete obliteration of one or more arteries and veins in each ear, with partial or local obliteration of segments of many of the remaining vessels. About 17 per cent of the animals showed marked constrictions in arteries or veins but no obliterative spasm. About 10 per cent showed no constrictions, but instead stoppage or slowing of the circulation. The remainder, about 23 per cent, gave no visible reactions at all. No spasm or constriction of vessels was ever observed in the scores of control, unsensitized mice, injected with the same sera.

In severe shock, when complete obliterative spasm occurred in both veins and arteries, almost no blood remained in the blanched ears. In mild shock general obliteration of the arteries and veins was absent, and the local constrictions trapped the blood in the unconstricted segments, as has been mentioned above.

It is a matter of much interest that, in occasional instances the veins constricted before the arteries and in a few instances of mild shock spasms appeared in the veins while the arteries never showed constrictions at any time. The findings show that the venous constrictions were not brought about by a lack of blood in the vessels. Indeed, as will be seen below, all the constrictive changes took place while the carotid blood pressure stood either at the initial normal level, or 10 to 40 mm. of mercury above it.

The Appearance of the Capillary Bed.—The appearance of the capillary bed differed much from animal to animal. To understand the differences it may be best, for descriptive purposes, to consider the vascular changes just described as falling into three general types; one in which arterial spasm set in before venous constriction; another, the commonest, in which arterial and venous spasm were synchronous; and finally, a relatively infrequent type characterized chiefly by venous spasm. Of course every possible combination of these types occurred, but as will be seen from what follows the happenings in the capillary bed were conditioned by the state of affairs in the larger vessels.

In most of the experiments in which there was obliterative spasm of the ear vessels the capillary bed blanched and the capillaries became invisible. This phenomenon occurred both in the instances in which arterial spasm preceded venous constriction and in those in which arterial and venous spasm occurred simultaneously. The capillary bed seemed to have emptied into the veins. An observer looking at the ear for the first time during this stage of shock might have readily assumed that an obliterative capillary contraction had occurred. However, this was not the case. In many experiments, one or two true capillaries (as defined by Chambers and his coworkers (20-23) were watched in one ear, at magnifications ranging from $\times 450$ to $\times 900$, while the changes in the larger vessels of the other ear were followed

through a low power ($\times 80$) microscope by another observer. When the capillary bed became blanched, as seen by the latter, the watcher at high power also lost sight of the capillaries he had been observing. Nevertheless, as he continued to search the spot where they had been visible, single blood cells suddenly appeared from time to time, passing rapidly through the invisible capillaries, which were obviously still patent but full of fluid. Presumably pressure from incompletely obstructed arteries skimmed off plasma which maintained a current flowing through the capillaries in which scattered blood cells moved on their way toward the veins.

When constriction of the veins occurred before that of the arteries, the true capillaries became choked with closely packed red blood cells. An observer seeing the ear at this stage for the first time might have remarked upon "capillary dilatation." However this is not the case; the capillary bed was simply pumped full with packed red cells.

It is clear from scores of such observations that the true capillaries of the mouse's ear are passive in anaphylactic shock; what happens in the capillary bed is determined by the site of spasm or constriction in the larger vessels. There is no contraction of the true capillaries, an observation which is in agreement with the recent findings of a number of workers studying other forms of shock (20-23).

The Recovery of the Circulation in the Ears.—In all mice that survived more than a few minutes, even in those that died after several hours and in those that, while surviving, yet showed complete obliterative spasm, recovery of the circulation in the ears began about $5\frac{1}{2}$ to 20 minutes after beginning the serum injection. The first movement of blood took place either in the veins or in the arteries. In instances in which both the capillary and venous beds were blanched, the earliest movement of blood began in the arterioles and was followed at once by a prompt surge of the cells into the already patent capillaries. In a surprisingly short time all the vessels in the ear became filled with blood and widely distended. In the instances in which the capillary bed was already filled with cells, the direct arteriolar-venular channels (the A-V bridges of Chambers and Zweifach (20-23) opened first and blood passed through them into the venules, regardless of whether the latter were filled or empty. As result, in all instances the venules became pumped full of blood cells as recovery progressed, and true capillaries leading to the venules often became even more distended by a reverse flow. Very rarely one saw a plug of well packed cells forcing its way through a minute vessel. The ears soon appeared in the gross as though in a state of flaming hyperemia, but under the microscope one could plainly see that the movement of blood was excessively slow. As will be seen below the systemic blood pressure during this stage was low.

Figs. 1 to 6 illustrate, at low magnification ($\times 25$), the changes that occurred in an ear during a moderately severe shock. The first photograph shows the ear before the serum injection, the second at one minute and a half after beginning it. Local obliterative spasms had occurred in practically all the vessels, trapping blood in them between the constricted segments. Fig. 3 shows the ear as it appeared 5 minutes after the injection. The arteries, together with many veins, had disappeared, but trapped blood can be seen in many other veins. In this instance complete obliterative spasm did not occur, but the ear, save for the trapped blood, was blanched and white and the capillary bed was empty. Fig. 4 shows early recovery, $12\frac{1}{2}$ minutes after the beginning of the injection. Veins here and there were filling with blood, but the arteries were scarcely

visible except under a higher power. Then one saw a hesitant, intermittent trickle of blood passing through them. Within 4 minutes more, however, (16½ minutes after the injection) all vessels in the ear had filled with blood (Fig. 5). The veins, distended almost to their initial calibers, were choked with closely packed, scarcely moving, blood cells. The capillary bed, too, had filled, but the arteries were still narrow and thread-like. Finally, half an hour after the injection (Fig. 6) the distended vessels gave to the ear the appearance of an intense hyperemia; but actually, for reasons to appear below, blood was scarcely moving through the organ.

In anticipation of findings to be given almost immediately below, it can be said that at the time that Figs. 2, 3, and 4 were taken, the carotid blood pressure was far above normal—and when the last two photographs were taken, it was far below normal.

THE BLOOD PRESSURE CHANGES AND THEIR RELATIONSHIP TO THE OTHER ANAPHYLACTIC PHENOMENA

It is generally believed that the clinical picture of anaphylactic shock results from some effect of the antigen-antibody reaction which leads, directly or indirectly, to constriction of smooth muscle in blood vessels, bronchioles, and other structures. A fall in blood pressure as a regular accompaniment of anaphylactic shock has been found in all animals in which suitable studies have been made. This has been ascribed by many to interference with the return of blood to the heart because of the constrictions of large veins in the abdomen or chest, a supposition substantiated by many *in vitro* experiments carried out with the Schultz-Dale technique and by the pathological findings in the lungs, liver, and other viscera in shocked animals. However in the later phases of anaphylactic shock there exists, along with low blood pressure, a profound vasodilatation. One would expect to find a compensatory vasoconstriction. Is the vasodilatation, therefore, an independent reaction, the result of the antigen-antibody reaction, or perhaps caused by nerve stimuli?

It is not known with certainty what rôle is played by the nervous system in the intact animal undergoing anaphylactic shock, nor what is the relationship between the vascular spasms, or subsequent dilatations, and the blood pressure changes. It seemed probable that the techniques for observing peripheral vascular changes here described, when combined with blood pressure measurement, might be used to answer some of these questions. We first determined what blood pressure changes, if any, occur in mice since this had not been previously studied. Next, after profound changes had been found, the time relationships between them and the onset of vasospasm and later dilatation were studied to learn which came first; that is to say, whether the vascular changes took place in compensation for the blood pressure changes or *vice versa*, whether they were occasioned by nervous stimuli, or whether they were independent reactions apparently brought about by the antigen-antibody reactions. The work has

shown that the vascular responses are independent of the blood pressure changes and furthermore are not determined by nervous stimuli.

Methods.—A previous paper from this laboratory (19) has described two methods for measuring blood pressure in the same mouse, one directly, by cannulation of the carotid artery, the other indirectly, by transillumination of the claws and observation of the blood flow in the claw bed, following inflation of a sphygmomanometer cuff placed about the thigh. Simultaneous measurements by both methods showed excellent agreement.

For the present work, mice, anesthetized with nembutal (19), were placed on their backs in the apparatus for measuring blood pressure (19), while their heads were supported in such a manner that the ears, with their dorsal surfaces facing downwards, spread out very lightly and without tension on glass slides. A few centimeters below the ears a mirror was fixed, and a strong cooled light, directed at an angle from below upwards, illuminated the dorsal surfaces of these organs, and allowed the brilliant reflexion in the mirror to be magnified by a binocular microscope. By these means the state of the blood vessels in the ear could be observed while blood pressure determinations were made at frequent intervals.

Thirty-two experiments were done. Eight normal mice were injected intravenously with 0.2 cc. of horse serum per 30 gm. of body weight. A second group of 16, sensitized to horse serum, were injected in the same way with similar amounts of the same serum. Of these, 11 showed spasms in the vessels of the ears. The remaining 8, sensitized to horse serum, were given intravenous injections of similar amounts of rabbit serum; that is to say, material containing proteins to which they had not been sensitized. In all, the systolic blood pressure was measured by both of the methods already mentioned (19) before, during, and after the serum injections, each of which required approximately 1 minute to complete.

Findings.—As already reported (19) the systolic blood pressure in the normal mouse anesthetized with nembutal or luminal, varies between 118 and 60 mm. of mercury depending largely upon the depth of the anesthesia. By the time that surgical anesthesia has been induced by the intraperitoneal injection of these anesthetics the systolic blood pressure is usually low, 60 to 90 mm. of mercury, and it may remain low for 15 to 45 minutes longer. As the anesthesia becomes lighter the pressure rises. Consequently in all the experiments to be considered here the pressure measurements were made at approximately the same time (45 minutes) after injecting the anesthetic. Only figures for the systolic pressure will be given hereafter.

Prior to the injections the systolic carotid blood pressure of all the mice ranged between 85 and 108 mm. of mercury. In some of them pricking the skin of the tail to inject the vein lowered the pressure by 5 to 8 mm. of mercury, but repeated prickings caused a rise of about 5 mm. Invariably the blood pressure was allowed to return to the previous level before the serum injections were begun. About 30 seconds after beginning the injections, when approximately half the dose had been given, the blood pressure began to rise by about 10 mm. of mercury. The rise continued to its maximum, usually 15 to 25 mm. above the preinjection level, in 1 to 3 minutes after the injection was completed. Occasionally the pressure rose by 30 to 40 mm. of mercury.

In the 8 normal mice, and in the 8 horse serum-sensitized ones injected with rabbit serum, there followed a gradual fall to the original pressure in the following 15 to 30 minutes. The blood vessels of the ears dilated slightly in some instances or remained unchanged while the circulation rate increased and the pulsation in the manometer in the carotid artery became greater.

Quite different were the findings in the 11 horse serum-sensitized mice which showed anaphylactic shock. In these the carotid blood pressure rose, as in the others, but at periods varying between 50 seconds and 3 minutes after the beginning of the injection, and either while the carotid blood pressure was still rising or while it remained at or near its maximum

height, spasm and constriction of the blood vessels of the ears took place. In several instances there was complete obliterative spasm of all the ear vessels and the organ was quite bloodless while the carotid pressure stood at levels 20 to 40 mm. of mercury higher than before the injection.

About 4 to 5 minutes after the beginning of the injection the blood pressure of the 11 mice that showed spasm of the ears began to fall while the spasm yet endured. Within 8 to 10 minutes the pressure usually reached the preinjection level, sooner than in the unsensitized mice or those receiving non-antigenic serum. One or 2 minutes later, when the pressure had reached a level about 20 mm. of mercury lower than the preinjection level, some of the ear vessels began to dilate and restoration of the circulation began. Thereafter the carotid blood pressure fell very rapidly, to 35 to 40 mm. of mercury, or even to 20 mm. or lower. The arteries and veins of the ears became more dilated, and the slow, stately flow of blood, already described, appeared in all of them. If death did not occur the blood pressure remained at levels between 20 to 45 mm. of mercury for an hour. Thereafter, further measurements by the direct method were abandoned.

Later Blood Pressure Changes. Studies of the Circulation in the Claws.—By the method for transillumination of the claws, already described (19), one observer watched the circulation in a claw bed of a hind toe, while another watched the mirror image of the ear vessels and noted the carotid blood pressure. The normal mice, injected with horse serum, and the horse serum-sensitized ones given rabbit serum, showed the usual rise in blood pressure, when the latter was measured in the leg, and also an increased circulation in the vessels of the claws. By contrast the circulation of the sensitized-shocked mice ceased at the same time that spasms began in the ear vessels, while the carotid blood pressure stood at the same high levels observed in the other animals which received serum injections, but in which there had been no blood vessel changes. The stoppage took place too rapidly to permit one to make pressure measurements by the indirect method. The blood cells in the claw bed remained immovable within the capillaries, which showed neither constrictions nor dilatations. When the circulation began again in the ears blood flow also reappeared in the claws of about half of the animals, indicating that in these instances spasm of the peripheral vessels in the ears and legs had relaxed simultaneously. In the remaining mice circulation in the claws reappeared later than in the ears, by periods ranging from 1 minute to 2 hours.

Since the blood flow in the claws ceased at the same time that vascular spasm occurred in the ears, the indirect method for measuring blood pressure in the legs was found useless during the acute stage of shock. However, animals with cannulated carotid arteries can be employed for limited periods only, and it was found possible to use the indirect method to advantage to study pressure changes occurring in the long period of recovery from shock. In 20 additional experiments blood pressure readings were made in the legs before shock was induced and again after shock, as soon as transillumination of the claws showed that blood flow had been resumed in the legs. The readings were repeated at intervals during the long period of recovery, or in the hours preceding delayed death, as the case might be.

When fatal shock occurred the blood pressure fell progressively to levels below 20 mm. of mercury and the animals died, either at once or within 1 to 3 hours. In the majority of survivors the pressure did not fall below 35 to 45 mm. of mercury, but even in these it required several hours to return to normal. The pressure of 4 of the 20 animals hovered between 18 and 30 mm. of mercury for 3 or 4 hours, the prostrated animals requiring no anesthetic or restraint while making the pressure measurements. Three of these died, but in the animal that survived the pressure began to rise slowly after the 3rd hour.

The findings show conclusively that the vasoconstriction in the ears and legs of the sensitized-shocked mice was not compensatory to a fall in blood pressure, since all the animals showed normal or elevated blood pressures at the time the

constrictions began. As shown by the experiments upon normal mice, part of the rise in blood pressure was due to the increase in blood volume occasioned by the injection of a relatively large amount of serum—0.1 cc. is equivalent to about 4 per cent of the blood volume of a 30 gm. mouse. The remainder of the rise in the shocked animals was conditioned, no doubt, by the closing off of many areas of the peripheral vascular bed by spasm of the arteries.

It is of interest that the carotid blood pressure began to fall while peripheral vascular spasm was still active, at least in the vessels of the ears. After the pressure had fallen to levels a little below the normal, vasodilatation took place, a reaction that can in no way be construed as compensatory in nature. It seems reasonable to assume that the vasodilatation was a primary reaction, and that it produced the fall in blood pressure. A vasodilatation occurring in the viscera or muscles could bring about the fall in carotid blood pressure even while the ear vessels remained in a constricted state. Work to be reported later has shown that such a vasodilatation does take place in the mouse.

THE PATTERN OF THE VASCULAR REACTIONS IS COMPLETELY INDEPENDENT
OF THE BLOOD PRESSURE CHANGES AND
OF NERVOUS CONTROL

Further experiments have shown more clearly that both the vasospasm and the subsequent vasodilatation of the ear vessels are wholly independent of the state of the blood pressure in the large arteries and that they are not brought about by nervous control.

It is generally believed that nerve stimuli are not of fundamental importance in the anaphylactic reaction. Seastone and Rosenblueth (24) have shown that the denervated nictating membrane of the cat contracts in anaphylactic shock. Lissak and Hodes (25) showed that anaphylactic shock of cats was similar, whether or not the sympathetic nervous system had been ablated, and Lissak and Kokas (26) found atropine to be without effect upon the progress of anaphylactic shock in dogs. These experiments, however, have ruled out special systems and specific mechanisms, not the nervous system as a whole, and additional evidence for or against the participation of the latter in anaphylactic shock is much to be desired. The techniques employed in the present work seemed to offer an excellent opportunity to throw light upon these questions.

Mice, sensitized and anesthetized as usual, were placed in plastaline moulds with only one ear lying on a porcelain plaque. The other ear was spread horizontally over a smooth cork which had been selected and carved for each experiment until it fitted perfectly into the auditory meatus of the mouse to be studied. The corks were supported in the plastaline moulds, and the pinnae lay smoothly over them with result that the blood flow in the slightly curved upper surfaces of the ears could be observed through the binocular microscope in the usual manner. A rubber band, 3 mm. wide, attached to a scale pan, was slipped over the ear supported by the cork and adjusted close to the ear base. While observing the circulation in the vessels, weights were added to the scale pan until the arterial circulation and all movement of blood in the ear ceased. Usually about 22 to 28 gm., inclusive of the weight pan, sufficed.

The usual shocking dose of serum was next given to the animal, and the vascular reactions were watched in both the obstructed and unobstructed ears.

The findings from 12 experiments of this sort have been summarized in Table I, with the individual instances arranged from the top downwards in relation to the speed at which spasm appeared in the unobstructed ear following the injection of the antigen. Complete obliterative spasm of the ear vessels occurred in 5 of the 12 mice (4+ in column b). In two other animals approximately three-quarters of the vessels went into spasm (3+), in 3 others half of the vessels (2+) were constricted, more or less, and in one, less than one-quarter (1+).

TABLE I

The Speed of Onset and the Intensity of Anaphylactic Vascular Reactions in Ears with Free Circulation and in Ears Subjected to Temporary Circulatory Obstruction during the First Phase of Anaphylactic Shock

In ears with free circulation			In ears subjected to temporary circulatory obstruction				
(a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)
Time to onset of vascular reactions*	Degree of spasm†	Period of recovery‡	Interval between recovery and re-release of block	Total period of block	Time to onset of spasm¶	Degree of spasm‡	Period of recovery**
min.		min.	min.	min.	min.		min.
0½	4+	10½	6	18	0½	4+	9½
0½	2+	11½	15	28	6½	2+	5½
0½	4+	16	60	77	4½	4+	12
1½	3+	13	15	30	1	3+	4½
1½	4+	10½	24	36	0½	3+	14
1½	3+	16	8	25	8	2+	16
1½	4+	17½	25	40	0½	2+	14½
1½	2+	7	19½	28	4	2+	12
2	2+	5½	5	12½	2	2+	5½
2	4+	10	3	14½	3½	2+	14
3½	1+	8	7½	16½	8	1+	17

One other animal showed no reaction in either ear.

* After the beginning of the injection of antigenic serum.

† See text.

‡ Time after the beginning of the injection, at which blood flow began again.

|| These figures represent the total period of circulatory block from the time that the rubber band and the weights were placed over the ear until they were removed.

¶ After the return of blood to the ear.

** The time after the return of blood to the ear.

In one mouse there was no reaction. No correlation appeared between the speed of onset of spasm and the intensity of the reaction. In all these animals the spasm of the vessels relaxed in 5½ to 17½ minutes after the injection began and recovery of the blood flow took place (Column c).

In no instances were any changes noted in the caliber of the vessels of the obstructed ears. Had nervous impulses initiated the constrictions in the unobstructed ears there should have been spasm of vessels on the obstructed side, for the periods of circulatory stoppage at the times that the vascular spasms

occurred in the unobstructed ears were not long enough—less than 4 minutes—to block nerve impulses, nor was the mild pressure of the rubber band sufficient to do so.

Final proof that the vascular reactions were completely independent of the blood pressure changes, indeed that they probably provoked the latter, was obtained in the following manner.

At intervals ranging from 3 minutes to $1\frac{1}{2}$ hours after recovery of the blood flow in the unobstructed ears the rubber bands were removed from the obstructed ones. At once blood flow commenced and in every instance except No. 12 (Table I) in which there was no reaction in the unobstructed ear, spasm and constriction took place in the experimental ear after the remainder of the animal's body had passed through its shock reaction. As the table shows (column e) the onset of spasm after the first influx of blood in the experimental ear was sometimes quicker, sometimes slower than in the other ear, and the time required for recovery differed somewhat. The intensity of the spasm in the two ears of the same animal also differed slightly but by and large the reactions were like those observed in the unobstructed ear, during the initial shock. It was obviously a local shock reaction, a replica of the preceding one in the other ear.

It is important to note that, after release of the circulatory obstruction, vasoconstriction took place while the animals' blood pressure must have been very low, which was always the case when measurements were made in the mice during the recovery phase. By contrast, when the constrictions took place in the unobstructed ears the pressure must have been higher than normal. The inference is clear, that the pressure had no effect upon the occurrence of the vasoconstrictions.

DISCUSSION

The chief finding of the present work would seem to be the occurrence of vasospasm and arrest of the circulation in the ears and feet of sensitized mice undergoing anaphylaxis. Such changes occurred not only while the blood pressure in the larger arteries was normal or slightly elevated, but at a time when unanesthetized animals, examined in the gross, showed either no symptoms whatever or at most only mild agitation and restlessness. The microscope revealed, both in unanesthetized and anesthetized animals, profound peripheral vascular effects imperceptible to the unaided eye. In the majority of cases the alteration was not fatal, and since it was to be perceived only by special methods, it can be termed occult anaphylaxis.

As already brought out, the constrictions and spasms of the ear vessels occurred only in sensitized mice, not in those previously normal. These reactions were primary phenomena in their own right and not dependent upon the changes in blood pressure or nervous stimuli. In mice poorly sensitized and showing little or no true shock at any time they were milder than in those

that passed later into severe or fatal shock, but the differences were of degree only, not of character. It is well known that sensitized or immunized mice yield notably poor skin reactions. This inference seems warranted, that the behavior of the ear vessels may be of service in the laboratory to detect slight degrees of anaphylactic sensitivity which might be missed by other means of investigation.

None of the earlier workers has studied the small vessels of the mouse during anaphylaxis. There has been no discussion of the reactions of minute peripheral vessels in sensitized animals not sufficiently hypersensitive to undergo actual anaphylactic shock. In two laboratories, however, constrictions and spasms of the vessels of the rabbit's ear have been observed during severe shock. Abell and Schenk (27) described them occurring within a Clark chamber in the animal's ear, and Bally (28-30) noted their appearance in the ears of rabbits during histamine, peptone, and anaphylactic shock. In this last and in histamine shock severe constrictions of the large arteries were seen early, about a minute and a half after the shocking injection, and after about 2 minutes, constriction of all the vessels took place. The blood pressure remained up until the spasms had reached their maximum, and fell thereafter, just as in our experiments on mice. By contrast, in peptone shock transient dilatation of the ear vessels occurred, followed by a late constriction after the blood pressure had fallen to its lowest point. The reactions witnessed by both groups of workers above mentioned must have been much like those which occur in the mouse.

Blanching in the ears of guinea pigs in anaphylactic shock has scarcely been mentioned in the literature, but a late cyanosis of the organs has been often reported. As is well known the systemic blood pressure first rises and then falls to low levels.

It would seem worthwhile to find out whether the ears of weakly sensitized rabbits and guinea pigs show peripheral vascular reactions in the absence of other signs of shock.

The present observations on mice showing severe shock have brought up some interesting possibilities. As already stated the behavior of the true capillaries of the ears was wholly unforeseen. They did not undergo any active constriction or dilatation, but simply remained patent and full of plasma though empty of red cells, or became crammed and distended with these latter, depending upon the state of affairs in the larger vessels. These findings fall in with those of recent workers (20-23) who have studied the behavior of true capillaries in other forms of shock.

The changes in the veins were pronounced, and the fact that they narrowed before the arteries did, or that in some instances constrictions occurred only in the veins, leads one to wonder whether something of the sort may not take place in skin during the development of wheals and other allergic skin phenomena in man.

It seems probable that the occurrence of occult anaphylaxis in generally sensitized animals showing no other signs of anaphylactic shock, and not locally sensitized, may have implications for the clinic. Allergists have long suspected that something like it happens in the sensitized tissues of man, and its occurrence would explain instances of partial collapse, distress, anxiety, and other symptoms for which no cause has as yet been found.

SUMMARY

Pronounced vascular changes occurring in the ears and claws of mice during anaphylactic shock are described. Practically at once after a foreign serum (pig, horse, or rabbit) enters the blood stream of sensitized animals both the arterial and venous vessels undergo marked, local or generalized constriction in the organs mentioned. Usually spasm of the vessel walls occurs simultaneously in the arteries and veins, but it may appear first in the arteries, or occasionally in the veins. When venous spasm precedes arterial spasm, the true capillaries become distended with cells; if the reverse order holds, the ears appear bloodless. There is no active constriction or dilatation of capillaries; the capillary behavior follows passively the changes in the large vessels.

Peripheral vascular spasm occurs while the carotid blood pressure is high, but a few minutes later, while this still holds true, the ear vessels begin to relax and the circulation is resumed. Shortly afterwards the blood pressure falls to levels far below normal, but the vessels remain open.

If the circulation of one ear is obstructed while anaphylactic shock is produced, no vascular spasm occurs in it. Release of the obstruction during the animal's recovery results in belated constriction of the blood vessels of this ear although by now the vessels in the other ear are dilated and the general systolic blood pressure is very low.

The vascular reactions in the ears appear to be uninfluenced by the blood pressure in the large vessels, and they are not a response to nervous stimuli. They are local in origin.

The vascular changes are often not clearly perceptible in the gross but are plainly to be seen under a low power of the microscope. They occur in some sensitized mice exhibiting no manifest signs of shock, differing only in degree from the changes taking place when shock is severe or fatal.

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EXPLANATION OF PLATE 29

The photographs were made by Mr. Joseph B. Haulenbeck.

FIGS. 1 to 6. Vascular changes in the ear of a mouse during moderately severe anaphylactic shock. $\times 25$.

FIG. 1. Before the shocking injection.

FIG. 2. $1\frac{1}{2}$ minutes after the beginning of the injection. Local obliterative spasms had trapped blood between the constricted segments.

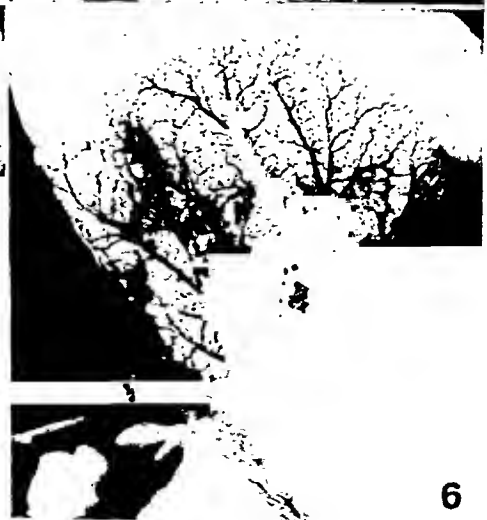
FIG. 3. 5 minutes after the injection. Most of the arteries and many veins had disappeared. In some veins trapped blood can be seen. The ear was blanched, the capillary bed empty.

FIG. 4. Early recovery, $12\frac{1}{2}$ minutes after the injection. The veins were filling with blood which had previously begun to trickle through the scarcely visible arteries.

FIG. 5. $16\frac{1}{2}$ minutes after the injection. All vessels of the ear were filled with slowly moving blood. The arteries were still narrow but the veins had reached their original calibers.

FIG. 6. Half an hour after the injection. The ear appeared intensely hyperemic, but the blood was scarcely moving in the vessels.

When the photographs for Figs. 2, 3, and 4 were taken, the carotid blood pressure must have been above normal—and when the last two photographs were taken it must have been far below normal.



(McMaster and Kruse: Vascular reactions in anaphylaxis)

QUANTITATIVE AIR-BORNE TUBERCULOSIS IN THE RABBIT

THE COURSE OF HUMAN TYPE INFECTION*

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PLATES 30 AND 31

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Lurie (1) has shown that by inbreeding it is possible to separate rabbits into families according to the degree of natural resistance they possess to a primary infection with bovine tubercle bacilli. While the differences are apparent after intracutaneous or intravenous infection, such artificial procedures are less informative than natural respiratory contagion from infected neighbors, such as occurs in man. By this latter method, however, the factor of dosage is uncontrolled and accordingly Wells and Lurie (2) resorted to quantitative air-borne infection, by means of the apparatus specially designed by Wells (3). When rabbits representative of the different families are exposed simultaneously to infection with a suitable dose of bovine bacilli, their differing resistance to pulmonary tuberculosis is seen in equally sharp relief. Hence it is justifiable to conclude that this resistance or susceptibility of rabbits to naturally or artificially acquired tuberculosis is a function of their genetic constitution.

Some progress has already been made by Lurie and his collaborators (1, 4-6) in analyzing the nature of this inherited resistance to acquired tuberculosis, but so far the work has been concerned solely with the disease as occasioned in rabbits by bovine tubercle bacilli. The greater resistance of the rabbit to human than to bovine infection is well known as a reliable means of differentiating these two types of tubercle bacillus. Hence it was considered most desirable to see what further light a study of human type tuberculosis, induced by quantitative, air-borne infection, would throw upon the nature of resistance in this animal. In order to define the variations that may occur under different experimental conditions it was decided to employ a heterogeneous group of rabbits, whose constitutional resistance to tuberculosis was unknown, as a preliminary to further investigation with inbred animals, the supply of which is limited.

*The expenses of the investigation were defrayed by a grant from the Commonwealth Fund.

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Material and Methods

Thirty-five rabbits were employed. They were purchased from several dealers, most being albino and the sexes mixed. In weight they ranged from 1800 to 3000 gm. at the time of infection, prior to which a tuberculin test was administered. All animals gave a negative reaction.

Quantitative air-borne infection was effected by means of a new apparatus, similar to that evolved by Wells (7) but incorporating a number of refinements which permit greater accuracy in the assessment of dosage. This most recent apparatus, together with a number of calibration experiments, is to be reported separately by Lurie *et al.*, but in principle the method is briefly as follows: A suspension of a young culture of *M. tuberculosis hominis* (H37Rv), grown on a modified Löwenstein's medium (8), is prepared in sterile M/15 Na₂HPO₄ so that the great majority of organisms exist singly. On the basis of opacity, photoelectrically determined, the suspension is diluted to a degree which previous experience with the apparatus has shown to give a suitable concentration of viable organisms in the infecting atmosphere. The suspension is atomized by compressed air to produce a bacterial aerosol in a specially designed flask. Here sedimentation of the coarser droplets occurs, whilst the finer droplets give rise to droplet nuclei. The latter pass into a long, wide-bore tube which carries them into a small infecting chamber from which they are conducted to a gas-operated incinerator. Adequate air flow through the apparatus is provided by an exhaust fan placed distally to the incinerator, the effluent passing out of the building. Only the heads of the rabbits project into the infecting chamber, which accommodates six animals at one time. The degree to which the animals are exposed to air-borne infection is determined by the concentration of viable organisms in the atmosphere of the chamber and by the duration of exposure. The former factor is assessed by taking at least two air samples of known volume from the chamber during the actual exposure, depositing the droplet nuclei in a known volume of suitable nutrient fluid, and then culturing the latter quantitatively on suitable media. For sampling a Wells air centrifuge is employed in conjunction with an inclined draught gauge, which measures the exact volume of air sampled. From the number of colonies that develop the number of bacterial units (so called because there is no means of knowing how many bacilli are included in a particular droplet nucleus) per litre of the air samples can readily be calculated, the average figure being taken to apply to the whole period of exposure. Kleiber (9) deduced a formula by which the amount of air inhaled per minute by laboratory animals can be derived from their body weights. This formula has been used to determine the amount of air inspired by each rabbit during the period of exposure and consequently the number of bacterial units inhaled can be estimated. Even with this apparatus a very high degree of accuracy in the control of dosage cannot be claimed. Accordingly the figures quoted should only be regarded as fairly close approximations to the truth. This, however, in no way invalidates the comparative value of the estimations.

Twenty-nine animals served for the study of the primary infection and six others were reinfected. In an attempt to follow the course of the ensuing disease weekly tuberculin tests were given as well as occasional x-ray examinations of the thorax. Old tuberculin, prepared from the same strain of organism as was used to infect the rabbits, was employed. The criteria adopted for a positive tuberculin reaction were those previously detailed (1). Radiography, as well as the tuberculin reaction after the initial positive response, proved to be singularly unreliable in this regard and thus arbitrary limits had to be set to the survival period of these animals. Three rabbits were killed before the time when the initial positive tuberculin reaction was expected, the remainder at intervals thereafter. During the experiment all animals gained weight and, excepting one (H25) that died from a non-tuberculous enteritis, all remained healthy until sacrificed by air embolism. Bacteriological procedures are indicated in their appropriate places below. For histological studies hematoxylin-eosin and Ziehl-Neelsen preparations from formalin-fixed, paraffin-embedded tissue were employed.

TABLE I

*Primary Quantitative Air-Borne Infection with H37Rn, Rabbits Showing Only Recent Lesions**

Rabbit No.	Estimated No. of bacilli inhaled	Old tuberculin reaction: time of onset after infection	Time of survival after infection	Pathological features		Presence of viable tubercle bacilli	
				Lungs	Hilar lymph nodes	In non-tuberculous lung	In hilar lymph nodes
		days	days				
H35	270	Neg.	8	No tubercles	No tubercles	+	+
H36	390	Neg.	13	No tubercles	No tubercles	+	+
H37	310	Neg.	20	No tubercles	Early tubercles	+	+
H24	440	18	27	1 early lesion in right lower lobe	Early tubercles	+	+
H8	470	22	31	1 early lesion in left lower lobe	Tubercles	+	0†
H33	2560	17	31	1 early lesion in left lower lobe	Tubercles	+	+
H34	2600	25	34	No tubercles	Early tubercles	+	+
H3	1750	34	38	3 early lesions in left upper lobe, right middle lobe, right lower lobe§	Early tubercles	+	+
H26	1210	29	50	2 early lesions in right upper lobe	Early tubercles	—	—
H17	1000	27	61	2 early lesions in left lower lobe	Tubercles	0	+
H23	1150	50	63	2 early lesions in left lower lobe, right upper lobe	Tubercles	+	+
H30	2400	25	84	3 early lesions in left lower lobe, right lower lobe, azygos lobe	No tubercles	—	—

* This table includes animals killed during the first 3 weeks after infection, before lesions had developed.

† Acid-fast bacilli present in section.

§ One tubercle in the spleen and two in the kidney.

RESULTS

I. PRIMARY INFECTION

Tuberculin Reaction

Excepting the three rabbits mentioned above, 25 out of 26 animals developed a positive tuberculin reaction (Tables I and II). This occurred 17 to 36 days after exposure to infection in 22 instances, while in two animals (H23 and H21) it was delayed until the 50th and 41st days, respectively, and in another (H14) it appeared as early as 11 days after exposure. As a rule the intensity of the

reaction increased to a maximum during the next 7 to 14 days and then gradually diminished, but in 5 instances the initial positive reaction was also the maximum one. After a further period of 7 to 77 days the tuberculin reaction became equivocal in most of the surviving animals, being neither a clear positive nor a complete negative (as in the preinfection test), but instead showing an area of erythema without significant induration. The reaction remained indefinite for the whole of the subsequent course of the experiment. Three rabbits (H7, H14, and H19), however, maintained a positive though fluctuating reaction until they were killed. One rabbit (H13) never gave a clearly positive response to old tuberculin, showing nothing more than the equivocal reaction after 18 days, but even this was greater than the negative preinfection test and it disappeared altogether after 60 days.

Response of the Host

Judging by the pathological appearances it was found that this series of rabbits could be divided into two main groups. In one of these all the lesions bore clear evidence of their recent development, while in the other group the changes were of considerably longer standing, or, as in several instances, completely lacking.

Animals with Only Recent Lesions.—From Table I it can be seen that early lesions alone are confined to those rabbits sacrificed no later than 84 days after infection. One to three primary lesions were present in any one rabbit. These had no predilection for any particular part of the lung, were subpleural in location, and usually projecting. They were spherical in shape, measuring 1 to 7 mm. in diameter, and were fairly well defined. Small foci of caseation were evident in some of the lesions (Fig. 1). Histologically the characteristic feature was the formation of large numbers of epithelioid cells with occasional giant cells, among which capillaries and occasionally a little hemorrhage occurred. Sometimes the epithelioid cells occurred in broad sheets obliterating the vesicular structure while in other cases they appeared to occupy alveolar spaces, the walls of which were still discernible. Surrounding and perhaps mingling with the epithelioid cells was a prominent inflammatory zone consisting of lymphocytes and plasma cells, among which polymorphs were sometimes numerous. Towards the centre of the epithelioid zone foci of necrosis were often present (Fig. 2). Fair numbers of tubercle bacilli usually occurred in the healthy epithelioid cells but they were more numerous in the caseous areas. Fibrosis did not occur. Neither tuberculous lesions nor tubercle bacilli were present in those parts of the lung remote from the primary lesions. The changes just described were present in 5 out of 6 rabbits killed shortly after their development of a positive tuberculin reaction (the single exception (H34) is discussed below) and in 3 further animals several weeks after the onset of allergic sensitivity.

The lesions of the latter 3 rabbits showed in addition evidence suggestive of a transition to a more chronic state in the form of metaplasia of the epithelium of the alveoli enclosed in the inflammatory zone to a cubical or low columnar type (Fig. 3). Dissemination was observed only once in the early stages of the disease (in H3, 38 days after infection), occurring *via* the blood stream to the spleen and kidney. No histological evidence of intrapulmonary spread was present in this group.

On gross examination of the tracheobronchial lymph glands no tuberculous lesions could be identified, although the glands were sometimes enlarged. Histologically, however, small epithelioid cell tubercles, containing few acid-fast bacilli were present in 8 of the 9 animals, the exception being that rabbit killed 84 days after infection. Little, if any, necrosis had occurred (Fig. 4).

The 3 rabbits killed before the time a positive tuberculin reaction was expected had no demonstrable evidence of tuberculosis, macroscopic or microscopic, in their lungs. One animal (H37) killed 20 days after infection did show early tubercles with acid-fast bacilli in its hilar lymph glands.

Animals with Long Standing Lesions.—Table II shows that such lesions were found only in rabbits surviving for 105 or more days. The old lesion resembled the recent one in shape and disposition, but was more sharply circumscribed. A typical old lesion was characterized by a large area of necrosis surrounded by a thin cellular zone (Fig. 5). The inner part of the latter consisted of epithelioid cells with occasional giant cells which rapidly merged with the caseous tissue. The outer part of the cellular zone was composed of chronic inflammatory cells in which a fine fibrosis could sometimes be detected, particularly subpleurally. Softening and cavitation were present in some lesions, while calcification was usually extensive in the caseous material, though also present to a lesser degree in the epithelioid cells (Figs. 6 and 7). Considering all the evidence it is justifiable to regard these old lesions as a late stage in the development of the primary lesions previously described. From one to four such lesions were present in 12 rabbits 105 to 207 days after infection. Six of these animals had in addition typical early lesions resulting from either local extension or bronchogenic dissemination while another rabbit showed extrapulmonary spread in the form of tubercles (containing acid-fast bacilli) in its appendix after 207 days. Local spread of the disease took the form of small close-set lesions in proximity to the primary one. Bronchogenic lesions were widespread, varied in size and number but were not necessarily associated with softening or cavitation of the primary lesion. Necrotic material containing acid-fast bacilli was present in a few bronchioles adjoining primary lesions. These 7 rabbits therefore showed a progressive form of disease. Five of the rabbits with chronic pulmonary lesions bore no evidence whatsoever of spreading disease 129 to 163 days after infection and their condition has been classed as stationary. Five other rabbits, 110 to

207 days after infection, showed no gross or microscopical evidence of disease but one of them (H13), never having developed a positive reaction to old tuber-

TABLE II

Primary Quantitative Air-Borne Infection with H37Rv, Rabbits Showing Old Lesions or None

Rabbit No.	Esti- mated No. of bacilli inhaled	Old tuberculin reaction; time of onset after infection	Time of survival after in- fection	Pathological features		
				Lungs		Hilar lymph nodes
				Old lesions	Recent lesions	
H27	1080	29	105	1 in left upper lobe*	3 in left upper lobe and right lower lobe	No tubercles†
H29	2240	17	110	None	None	No tubercles
H7	1360	17	127	2 in left lower lobe and right upper lobe	Numerous scattered lesions of broncho- genic origin	Few tiny tubercles
H31	2370	17	129	1 in left lower lobe	None	No tubercles
H32	2180	25	129	None	None	No tubercles
H25	1080	21	142	2 in left upper lobe and right lower lobe	None	No tubercles
H28	1100	36	150	2 in right upper lobe and right lower lobe‡	Many of local origin	No tubercles§
H20	810	33	152	4 in right upper lobe, right middle lobe, and right lower lobe	None	No tubercles
H21	730	41	152	None	None	No tubercles
H18	1060	20	163	1 in right middle lobe	None	No tubercles
H19	920	27	163	3 in right upper lobe, right middle lobe, and right lower lobe	None	No tubercles
H22	870	27	163	2 in left upper lobe and right middle lobe	Many of local origin	No tubercles
H12	580	32	207	1 in left lower lobe	2 in left lower lobe	No tubercles
H13	480	Equivocal only after 18 days	207	None	None	No tubercles
H14	510	11	207	1 in left upper lobe¶	None	No tubercles
H15	570	32	207	4 in left upper lobe, left lower lobe, and right middle lobe	3 in right middle lobe	No tubercles
H16	590	25	297	None	None	No tubercles

* Viable bacilli in non-tuberculous lung (by guinea pig inoculation).

† No viable bacilli in hilar glands (by culture).

‡ No viable bacilli in non-tuberculous lung (by guinea pig inoculation).

§ Viable bacilli in hilar lymph nodes (by culture).

¶ Tubercles, with acid-fast bacilli, in the appendix.

culin, cannot be regarded as having had a primary lesion (see below) and so must be excluded from this group of animals whose disease had retrogressed.

All but one of these 16 rabbits showed no microscopical involvement of the hilar glands by tuberculosis. In the one exception (H7) a few tiny foci of epithelioid cells were present but no acid-fast bacilli could be demonstrated. In one other rabbit (H28) with negative histological findings, tubercle bacilli were recovered on culture of the glands.

Fate of the Bacilli

In the Primary Lesion.—This question was approached by quantitative culture of representative portions of primary lesions from rabbits sacrificed at different intervals after simultaneous infection. Only 3 of the 5 animals originally infected proved suitable for cultural purposes. After aseptic autopsy approximately half of the primary lesion was excised and a uniform suspension (1:1000) prepared in M/15 Na_2HPO_4 . A portion of this "direct" suspension was treated with sulfuric acid and both were cultured quantitatively on three tubes of Löwenstein's medium and three of Dorset's. In addition portions of all hilar glands were cultured qualitatively.

The results are given in Table III and these suggest that first there is a proliferation of tubercle bacilli in the primary lesion, since all the organisms living there in H23 could not have been deposited at the time of exposure. Subse-

TABLE III
The Fate of the Bacilli in the Primary Lesions and the Hilar Lymph Nodes

Rabbit No.	Estimated No. of bacilli inhaled	Time of survival after infection	No. of primary lesions	No. of bacilli per mg. of primary lesion	Recovery of bacilli from hilar lymph nodes
		<i>days</i>			
H23	1150	63	2	5900	+
H27	1080	105	1	130	0
H28	1100	150	2	1300	+

quently the number of organisms per unit weight of lesion, as revealed in H27 and H28, diminishes considerably. The cultural results were corroborated by visual comparison of the numbers of bacilli in sections from the same lesions. Since the observations are so few the conclusions can only be tentative, especially as the rabbits' hereditary resistance was unknown and this may well have an important influence on the fate of the bacilli. That the latter factor is not very dissimilar in the 3 animals is suggested by the facts that all developed rather large primary lesions and that in the 2 surviving sufficiently long for secondary changes to occur both showed softening of the primary lesion with local extension of the disease, the latter being more advanced in the longer survivor. Nevertheless the procedure should be repeated using litter mates of resistant and susceptible families of rabbits. It is interesting to observe that tubercle bacilli were recovered from the hilar glands only when considerable numbers of organisms occurred in the primary lesion(s) and that no histological evidence of tuberculosis was found in the hilar glands of H27 or H28.

In Lung Tissue Remote from the Primary Lesions and Showing No Gross Abnormality.—Lurie (1), using bovine bacilli, demonstrated that a large proportion of the bacillary units inhaled produced tuberculous foci. As opposed to this

human bacilli induce very few lesions in proportion to the number inhaled. The question thus arose as to the fate of human bacilli which reached the lungs but did not produce a response on the part of the host. From each of 12 rabbits, 8 to 150 days after exposure to infection, several grams of lung tissue were excised aseptically and carefully cut into tiny pieces. The latter procedure excluded any lesion visible to the naked eye and this was later confirmed by histological examination of precisely similar lung tissue. A suspension of the lung was prepared and either cultured or inoculated into a guinea pig. From Tables I and II it can be seen that viable tubercle bacilli were recovered on ten out of twelve occasions and for as long as 105 days after exposure to infection.

In the Tracheobronchial Lymph Glands.—Tables I, II, and III show that in ten out of eleven cases culture of the hilar glands revealed tubercle bacilli and for as long as 150 days after infection. In one further case the stained section showed acid-fast bacilli in tubercles in these glands. The histological response was always of a minor degree and in three instances where viable bacilli were found, no tissue response was apparent 8, 13, and 150 days after infection. In two of these, however, it is likely that sufficient time had not elapsed for tissue changes to develop.

II. REINFECTION

Table IV gives the results of a single reinfection with H37Rv in six instances. One rabbit (H6) is not regarded as having developed a primary lesion from the first exposure, judging by its failure to show a positive tuberculin reaction then and the fact that on reexposure the reaction pursued a course characteristic of a primary infection. The reason for this failure is unknown. Despite a reinfecting dose of over 2000 bacilli, 73 to 87 days after the primary exposure, none of the rabbits showed lesions which could be attributed to the reexposure nor was the course of the disease induced by the primary infection modified in any detectable way. These results are in accord with those of Lurie (10) who found that after intravenous reinfection of rabbits with human tubercle bacilli no gross lesions attributable to the reinfection developed in the presence of residual primary lesions. Excluding H6 the tuberculin reaction had become equivocal before the reinfection, but thereafter returned to a positive response for a brief period, though of less intensity than that following the primary infection. These observations may be interpreted as meaning that the bacilli of reinfection, in the doses employed, do evoke some response in the host, but that they are then rapidly and completely destroyed.

DISCUSSION

Pathologically the course of the disease induced by quantitative air-borne infection of rabbits with human tubercle bacilli appears to be as follows: For about 3 weeks after exposure no detectable response occurs on the part of the

host. Subsequently one or more primary lesions are recognizable and for as long as 12 weeks after infection the histological features indicate their recent origin. Beyond this period the disease pursues one of three courses. Firstly, the primary lesions may retrogress and ultimately disappear, thus representing healing of the disease. Baldwin and Gardner (11) using R1 bacillus infection in guinea pigs saw primary lesions in the process of resorption and finally heal-

TABLE IV
Quantitative Air-Borne Reinfection with H37R₀

Rabbit No.	Estimated No. of bacilli inhaled		Primary old tuberculin reaction; time of onset after infection	Time of survival after:		Pathological features	
	Primary infection	Reinfection		Primary infection	Reinfection	Lungs	Hilar lymph nodes
H2	1900	2540	days 27	days 136	days 49	1 old lesion in right lower lobe. Numerous recent lesions scattered throughout both lungs	No tubercles
H4	1710	2420	27	205	118	No tubercles	No tubercles
H6	390	2230	Negative after primary ? infection; positive 25 days after re-infection	219	146	2 old lesions in left lower lobe and right middle lobe. Recent lesions in left upper lobe, left lower lobe, right upper lobe, right lower lobe, azygos lobe	Few early tubercles with occasional bacilli
H9	450	2590	28	219	146	No tubercles	No tubercles
H10	440	2390	28	259	186	1 old lesion in left lower lobe. Small recent lesions of local origin	No tubercles
H1	1890	2660	21	273	186	No tubercles	No tubercles

ing, but such was not observed in this series and the evidence for complete retrogression is given below. Secondly, the primary lesion may remain and develop features indicative of chronicity, yet showing no tendency to spread locally or *via* the air passages. Such lesions have been described as stationary but their ultimate fate is not yet certain. Absorption with resolution seems improbable on histological grounds and the prominence of calcification suggests that it may be the terminal state. That progression may nevertheless occur is shown by the alimentary spread from an isolated and apparently stationary

pulmonary lesion (H14). The third possibility is that the primary lesion may give rise to progressive pulmonary disease either by local extension or bronchogenically. Had those rabbits which exhibited this form of disease been allowed to survive indefinitely it is most likely that they would have succumbed to extensive pulmonary tuberculosis. Hematogenous dissemination occurred in only one instance (H3) and that at an early stage of the disease.

A striking feature in this series of rabbits was the absence of any macroscopical involvement of the hilar glands. With one exception microscopical tubercles in these structures were confined to animals with recent primary lesions in their lungs. Viable bacilli were present before tubercles could be detected and also in one instance (H28) after apparent retrogression of the tubercles. Lurie (1) observed that with bovine infection of naturally resistant and susceptible animals only the latter developed gross disease of their hilar glands. In the rabbit the general level of resistance to human infection is higher than that to bovine, so the present findings support previous ones. Few bacilli could be seen in the glandular lesions and here lies the probable explanation for the paucity of the reaction. In turn this suggests that dissemination by way of the lymphatics from the primary lesion is restricted, as would be expected if resistance to the disease is due basically to localization of the infection to the portal of entry (1).

The development of an undoubted positive reaction to old tuberculin 3 to 4 weeks (on the average) after infection was associated with the persence of one or more early pulmonary lesions in 8 out of the 9 rabbits sacrificed within the first 12 weeks of the experiment. Of these 9 animals 6 were killed a few days after the tuberculin reaction first became positive. Rabbit H34 was exceptional in that no gross lesion was recognized in the lungs despite a positive tuberculin reaction, the recovery of bacilli from the lungs, and the occurrence of tubercles with viable bacilli in the hilar lymph glands. The most probable explanation is that, despite close scrutiny, a small primary lesion was overlooked, especially as the findings in this animal differ from those in H37 only in the response to tuberculin (Table I). Accordingly a positive tuberculin reaction can be regarded as signifying the development of a pulmonary lesion and, in the latter, caseation is usually, but not necessarily, present. It is thus safe to say that those rabbits whose positive reactions later subsided did have a primary lesion originally and that the absence of gross disease at autopsy implies that such lesion resolved completely or retrogressed sufficiently as not to be visible to the naked eye. Lurie (12) also encountered positive tuberculin reactions in some rabbits exposed to natural contagion with bovine bacilli and in which no evidence of tuberculosis was found at subsequent autopsy. He considered the tuberculin reaction to be specific and to indicate the penetration into, and interaction with the tissues of tubercle bacilli in sufficient concentration to induce an allergic state. It is well recognized that in man, as also in experimental ani-

mals, the tuberculin reaction may wane in intensity as the primary infection retrogresses (13-16) and with this the present findings agree. In the majority of the rabbits in this series, however, waning occurred in association with progressive and stationary tuberculosis. It is impossible to say whether this desensitization was specific or not (15). Lurie (1) observed that resistant animals gave a less intense reaction to tuberculin than susceptible ones after infection with bovine bacilli and this parallels the greater sensitivity to tuberculin of the Negro, who is susceptible to tuberculosis, than of the white, who is more resistant. It would be expected that rabbits, being generally more resistant to human than bovine tuberculosis, would show a less intense tuberculin reaction in the former type of disease than in the latter. This in fact was observed, the reaction being more extensive, indurated, and erythematous as well as sustained, following bovine infection. This phenomenon may be interpreted as meaning that the rabbit localizes the infecting human bacilli more effectively

TABLE V

Relationship of Primary Infecting Dose to the Subsequent Course of the Disease

Estimated dose (approximate)	No. of rabbits showing:			
	Recent Lesions only	Progressive disease	Stationary disease	Retrogressive disease
500	2	3	0	2
1000	3	4	4	0
2000 and over	3	0	1	2

to the site of deposition or that it destroys the bacilli and their products more rapidly and effectively, so hindering the entry of sensitizing antigens into the circulation.

Whether human type tuberculosis in the rabbit progresses or not depends on two possible factors, the dosage of the infecting organism and the natural resistance of the animal. The rabbits in this series were exposed in groups of 4 to 6 and it can be seen from Tables I and II that the estimated infecting dose varied considerably in different animals. In Table V the course of the disease has been arranged according to the dose of bacilli. Within the limits of these experiments dosage does not appear to determine the number of primary lesions which develop nor whether the latter progress or retrogress. In fact, no example of progression occurred with the highest scale of dosage. This contrasts with the previous experience of bovine tuberculosis in which there appears to be a correspondence between the number of organisms estimated as inhaled and the number of bacilli subsequently recovered from the lungs, as well as with the number of primary tubercles engendered and the eventual progress of the disease (1, 19). The conclusion from these experiments must therefore be that

the determining influence on the course of the disease is the degree of natural resistance to the infection.

The occurrence of viable tubercle bacilli in lung tissue apparently devoid of any pathological changes for as long as 15 weeks after infection has a parallel in human pathology. Opie and Aronson (17) recovered by guinea pig inoculation tubercle bacilli from lung tissue unaffected by tuberculosis in twelve out of thirty-one miscellaneous autopsies on individuals dying from causes other than tuberculosis. All twelve cases, however, bore evidence of a previous pulmonary infection as indicated by quiescent fibrocaseous lesions in the lung, pleural scars, or calcified glandular lesions. Later Aronson and Whitney (18) showed that in six of the twelve cases previously reported the tubercle bacilli were all of human type. In three of these six cases the lung tissue was taken from the apex of the lung but in the remainder it came from the base. The viable tubercle bacilli in normal lung tissue may have been derived from the tuberculous lesions in other parts of the same lung and this may be true of the experimental disease, but it is also possible that in the rabbit the organisms recovered from apparently normal lung were deposited at the time of the exposure to infection. Judging by the lesions in the longest survivors these bacilli must eventually die, but the possible significance of repeated exposures at short intervals after a primary one in building up the local concentration of organisms from a subinfective to an infective level must not be overlooked. Clinical experience, especially with children, is in accord with this view.

SUMMARY

1. Pulmonary tuberculosis in unselected rabbits, induced by primary quantitative air-borne infection with human type tubercle bacilli, may retrogress or progress. Some animals whose disease was in a stationary condition might have fallen into one of the above groups had the experiments been prolonged.

2. Within the limits of the observations natural resistance or susceptibility appears to be the chief factor in determining the course of the disease.

3. Following the development of the primary lesions the tuberculin reaction became positive but thereafter proved to be an unreliable indicator of the course of the disease.

4. Tubercle bacilli can be recovered from macroscopically normal lung tissue of rabbits several weeks after primary infection.

5. Reinfection did not induce the formation of new lesions nor alter the course of the disease caused by the primary infection.

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EXPLANATION OF PLATES

All microphotographs were prepared from tissues fixed in formalin and imbedded in paraffin and stained with hematoxylin-eosin.

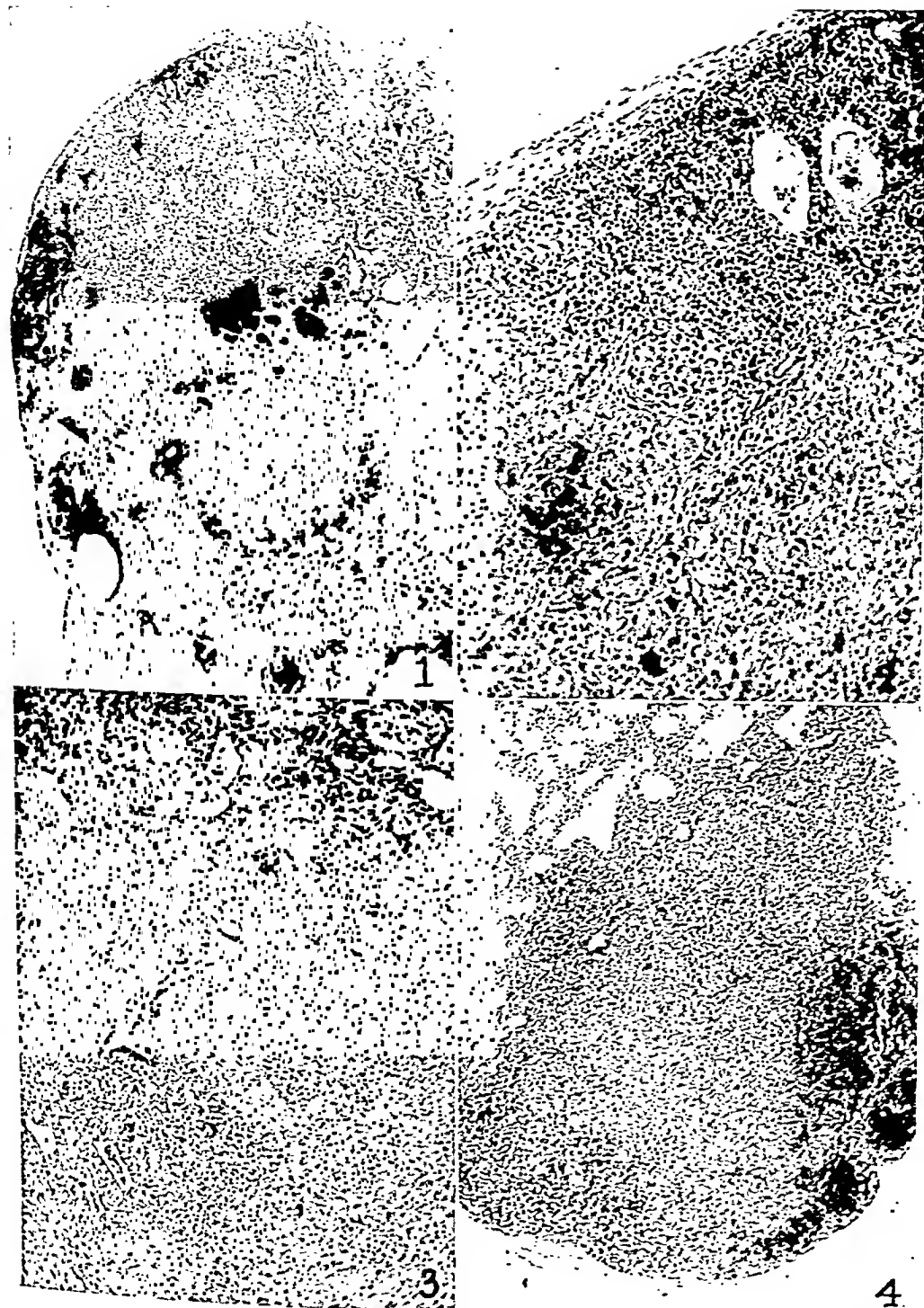
PLATE 30

FIG. 1. Early primary lesion in rabbit H3, 38 days after a primary infection. Subpleural mass, mainly of epithelioid cells, not very well defined. Central areas of caseation (very dark in photograph). × 24.

FIG. 2. Early primary lesion in rabbit H3, 38 days after a primary infection. Subpleural lymphocytes bordering epithelioid cells with one focus of commencing necrosis. × 145.

FIG. 3. Recent primary lesion in rabbit H17, 61 days after a primary infection. Alveolar metaplasia in the wide inflammatory zone. Epithelioid zone thinned and necrosis more extensive. × 92.

FIG. 4. Hilar lymph node in rabbit H23, 63 days after a primary infection. This pale mass of epithelioid cells with commencing necrosis represents the most advanced degree of change seen in any hilar lymph node. × 46.



(Heppleston: Quantitative air-borne tuberculosis in rabbit)

PLATE 31

FIG. 5. Old lesion in rabbit H31, 129 days after a primary infection. Inflammatory and epithelioid zones now reduced in width. Calcification of the large caseous area prominent. Whole lesion sharply circumscribed. $\times 55$.

FIG. 6. Old lesion in rabbit H15, 207 days after a primary infection. Subpleural fibrosis. Very narrow cellular zone with calcification. Latter more evident in caseous material. $\times 110$.

FIG. 7. Old lesion in rabbit H20, 152 days after a primary infection. Delicate fibrosis of the wall with relatively scanty cellular zone and extensive calcification, especially of the caseous tissue. $\times 150$.



THE EFFECT OF ALLOXAN DIABETES ON EXPERIMENTAL CHOLESTEROL ATHEROSCLEROSIS IN THE RABBIT*

I. THE INHIBITION OF EXPERIMENTAL CHOLESTEROL ATHEROSCLEROSIS IN ALLOXAN DIABETES

II. THE EFFECT OF ALLOXAN DIABETES ON THE RETROGRESSION OF EXPERIMENTAL CHOLESTEROL ATHEROSCLEROSIS

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The problem of arterial disease associated with diabetes mellitus has loomed larger and larger ever since the discovery of insulin. However, experimental study of this problem has been thwarted by the fact that dogs and cats, in which experimental diabetes can be readily produced by several methods, are notoriously resistant to the development of arterial disease. On the other hand, although experimental arterial diseases of various types can be produced very easily in rabbits, this species cannot be rendered permanently diabetic by anterior pituitary extracts and its pancreatic tissue, like that of other rodents, has an anatomical distribution such as to render total pancreatectomy an operation of extreme technical difficulty. Thus, prior to the recognition of the diabetogenic properties of alloxan, it was not feasible to study experimentally the effects of diabetes in the one mammalian species that appeared most likely to respond to such a metabolic disturbance with the development of some form of arterial disease. The discovery that rabbits can be rendered permanently diabetic by alloxan has made it possible to embark upon a study of the effects of diabetes on the arteries of rabbits with the hope of gathering experimental data that might have significance not only in relation to the problem of arteriosclerosis developing in man in association with diabetes mellitus but also in relation to the larger problem of arteriosclerosis in general.

The pathological changes encountered in the pancreas and in certain other tissues of animals rendered diabetic by pancreatectomy, by the administration of anterior pituitary extracts, and by the injection of alloxan have been reviewed elsewhere(1). In some of the earlier papers dealing with alloxan diabetes as observed in various species, it was stated that no changes in the arteries were found in association with this experimental disease in spite of the coexistence in some instances of marked

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lipemia, but the duration of these experiments was relatively short. The occurrence of visible lipemia in alloxan diabetes in rabbits lent support to the supposition that atherosclerosis might develop in them after a sufficient period of time, since it is well known that the continued feeding of cholesterol to rabbits is followed by the appearance of lipemia, hypercholesterolemia, and the eventual development of experimental cholesterol atherosclerosis(2-4). Accordingly, Duff and Wilson(5) carried out a series of experiments in which the blood cholesterol levels were followed during the course of prolonged alloxan diabetes in rabbits with a view to determining at the end of the experiments whether any effect had been produced on the arterial system. They found that lipemia was usually an evanescent phenomenon and in some cases did not occur at all. The blood cholesterol was frequently elevated for a time to values of about 350 mg. per cent but it almost always returned to normal in 3 to 6 weeks and so remained for the rest of the animal's life, in spite of the persistence of more or less severe diabetes as judged by the continuance of marked hyperglycemia, glycosuria, polydipsia, polyuria etc. In these experiments they could find no evidence at autopsy of the development of atherosclerosis either in the aorta or other arteries of rabbits that had been diabetic for periods of time ranging from several months up to a maximum of 1 year.

In view of the negative result just described, the experiment reported in Part I of the present communication was undertaken. It was designed to permit of a comparison between the effects of cholesterol feeding in normal rabbits and in rabbits previously rendered diabetic by alloxan. The unexpected result of this experiment was the demonstration that the development of cholesterol atherosclerosis is markedly inhibited in alloxan-diabetic rabbits as compared with non-diabetic control animals, in spite of the fact that hypercholesterolemia of comparable degree is induced in the two groups of animals by cholesterol feeding. This result has already been briefly recorded (6).

The inhibition of the development of experimental cholesterol atherosclerosis in rabbits rendered diabetic by alloxan suggested the importance of determining whether the retrogressive changes that are known to occur in the arterial lesions after the feeding of cholesterol is terminated (2, 3) would be affected by the induction of alloxan diabetes at the completion of an adequate course of cholesterol feeding. An account of an experiment carried out with this end in view is contained in Part II of the present paper.

I. THE INHIBITION OF EXPERIMENTAL CHOLESTEROL ATHEROSCLEROSIS IN ALLOXAN DIABETES

Materials and Methods

The animals employed were young, adult, domestic white rabbits of both sexes. The ages of these animals were unknown. They weighed from 2 to 3 kilos at the beginning of the experiment, and all were healthy, growing animals. Housed in separate metal cages, they were given a diet of Purina rabbit chow and water *ad libitum*. No dietary supplements were used. The animals were divided into convenient groups or series, and after a period of acclimatization of 1 or 2 weeks determinations of the content of sugar in the blood and of

free and total cholesterol in the serum were made in the fasting state by a modification of Folin's micro method, and by the Schoenheimer and Sperry method respectively. After establishing that these quantities were within normal limits, half of the animals were selected at random and were injected with a freshly prepared 5 per cent aqueous solution of alloxan (Eastman Kodak Co.) in the lateral ear vein. The dose was 200 mg. per kilo of body weight. This treatment was followed by the daily injection of 2 to 6 units of protamine zinc insulin and of about 1 gm. of dextrose for a period of 7 to 14 days. The animals were then left without further treatment for a period of 4 to 5 weeks in order to allow for stabilization of the metabolic processes, after which cholesterol feeding was instituted. Urinary sugar and acetone estimations were made when indicated. Fasting blood sugar and cholesterol values were estimated at approximately biweekly intervals.

The normal control and alloxan-injected animals in each series were fed exactly the same doses of cholesterol on the same days of the experiments and, except for the previously mentioned period of insulin and dextrose therapy, were treated exactly alike. The animals of series 3 received dry powdered cholesterol in gelatin capsules, according to the method of Pollak (7). The animals of all other series received a 3.3 or 5 per cent solution of cholesterol in corn oil dissolved and maintained at 60°C. and fed by means of a stomach tube after cooling. The daily dose of cholesterol varied in the different experimental series from 0.25 to 0.75 gm. The details of the total amounts of cholesterol fed, and the durations of feeding in the various experimental series are shown in Table I.

On completion of the period of cholesterol feeding the surviving animals were killed by air embolism, complete autopsies were performed, and the tissues were fixed and sectioned. The aorta and heart were removed *en bloc*, fixed in formalin, stained in Sudan IV, and the fatty deposits in the intima revealed by this method were recorded on standardized, schematic drawings of the aorta. The aorta was then severed from the heart, small blocks were removed for frozen sections, and the remainder was rolled into a coil and embedded in paraffin. These blocks were sectioned in such a manner that a single microscopic section included the entire length of the aorta. The paraffin sections were stained with hematoxylin and eosin, Verhoeff-Van Gieson stain for elastic tissue, and Mallory's phosphotungstic acid hematoxylin. Frozen sections of the aorta, liver, spleen, and adrenal gland were examined after staining with Sudan IV.

The maximum values accepted as normal were: blood sugar 160 mg. per cent, total cholesterol 80 mg. per cent, and free cholesterol 35 mg. per cent. An animal was regarded as being diabetic if the fasting blood sugar average was 300 mg. per cent or greater, and if most of the obvious manifestations of alloxan diabetes, such as persistent polyuria, glycosuria, polydipsia, polyphagia, and weight loss were present. In addition, the histological demonstration of the characteristic hydropic changes in the pancreas (8) and the lesion of Armani in the kidney were taken as confirmatory evidence of the existence of a persistent diabetic state. Certain of the animals that had received a diabetogenic dose of alloxan, although they were initially diabetic, reverted to a normal metabolic state by the time that cholesterol feeding was instituted and thereafter showed neither chemical nor obvious evidence of diabetes. Such animals were classed as "alloxan-recovered" (see Table I). The degree of experimental cholesterol atherosclerosis observed in each animal was graded on an arbitrary scale of 0 to 4 as shown in Fig. 1. It is important to note that atherosclerosis of grade 1 severity was recorded even when only a single fleck of lipid deposit was revealed by careful gross examination after Sudan staining of the entire aorta. This gross grading was confirmed by microscopic examination. The amount of sudanophilic, lipid substance in the liver, spleen, and adrenal was graded in a similar arbitrary manner on a scale of 0 to 4 by microscopic examination of stained, frozen sections. A lipemic condition of the blood induced by the diabetic state and/or cholesterol feeding was similarly graded by inspection of the blood

before and after clotting *in vitro*. A lipemic index for each animal was established by averaging the grades of lipemia observed on all the occasions when the blood was sampled.

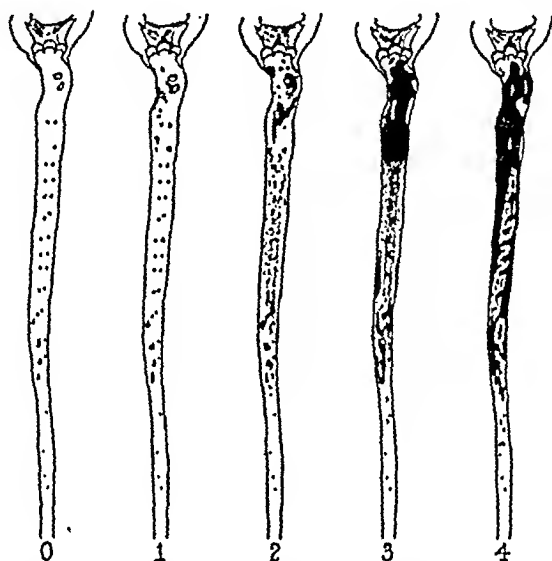


FIG. 1. The standard schematic diagram of the rabbit's aorta used for charting the extent of the atherosclerosis observed at autopsy is illustrated. The five diagrams represent the aortic lesions observed in five animals and show, from left to right, the degrees of atherosclerosis graded as 0, 1, 2, 3, and 4 respectively.

Observations

The observations are summarized in Table I. Over 100 animals were used in this experiment, but the mortality rate from the use of alloxan and from feeding and other accidents was high. Only the 58 animals that completed an experimental course of satisfactory duration are reported upon. These comprised 18 diabetic and 39 non-diabetic animals. In addition, there was one animal (No. 43) that showed a moderate elevation of the blood sugar level in the postprandial state only, and is referred to in the table as mildly diabetic. The 39 non-diabetic animals comprised 13 rabbits that had recovered from the effects of a diabetogenic dose of alloxan ("alloxan-recovered" group), and 26 normal control animals that were subjected to cholesterol feeding alone. The period of cholesterol feeding varied from 52 to 91 days. The total dose of cholesterol fed varied between 16 and 65 gm. and the average daily dose of cholesterol fed varied from 180 to 750 mg. among the different experimental series. The chemical values given in the table are arithmetical averages of biweekly determinations made during the period of cholesterol feeding and of one determination made immediately before cholesterol feeding was begun. The very high maximum values obtained in some of the animals are not shown, but are, nevertheless, indicated by the average values recorded.

The dosage of cholesterol and the duration of cholesterol feeding varied from those that induced neither a hypercholesterolemia nor cholesterol atherosclerosis among the control animals (series 1 and 2) to those that induced in the controls a very severe degree of both, with the formation of confluent atherosclerotic lesions extending over the entire intimal surface of the aorta and measuring from one to one and one-half times the thickness of the underlying media. However, inspection of the data in Table I reveals that there was a remarkable discrepancy in the degree of atherosclerosis induced in the diabetic animals as compared with that induced in the comparable groups of control rabbits fed exactly the same quantities of cholesterol within the same period of time (Figs. 2 and 3). In each experimental group, with the exception of series 1 and 2 in which no atherosclerosis was induced in any of the animals, the diabetic rabbits showed a striking resistance to the induction of experimental cholesterol atherosclerosis of the aorta. On the other hand, the control rabbits and the "alloxan-recovered" group presented the expected incidence and degree of cholesterol atherosclerosis without distinction. The 23 control animals contained in series 3 to 11 inclusive, all presented more or less severe atherosclerosis of the aorta. Of these 23 animals, 8 presented aortic atherosclerosis of grade 1 severity, 4 of grade 2, 4 of grade 3, and 7 of grade 4. The 12 "alloxan-recovered" animals in these same series presented similar findings. Of these 12 animals 1 showed no atherosclerosis, 2 had aortic lesions of grade 1 severity, 4 of grade 3, and 5 of grade 4. In contrast to these results, the 13 diabetic animals in the same groups presented no atherosclerosis in 6 animals, grade 1 aortic lesions (usually minimal) in 6, and aortic atherosclerosis of grade 2 (minimal) in only 1 rabbit. The quantities of sudanophilic lipid material present in the liver, spleen, and adrenals, in general, corresponded closely with that found in the aorta. The diabetic rabbits showed evidence of the same resistance to lipid deposit in the liver, spleen, and adrenals as in the aorta. Indeed, some of the diabetic rabbits were completely protected from the deposition of cholesterol, as judged by careful morphological examination, not only in the aorta but also in these other organs.

Most important is the observation that the diabetic state did not inhibit the development of a hypercholesterolemia that was as high as that induced in the corresponding "alloxan-recovered" and control animals and that was occasionally considerably higher (Figs. 2 and 3). This was also true of both the free and ester fractions of the serum cholesterol; there was no significant alteration in the ratio of ester to total cholesterol content. In addition to exhibiting an equally marked degree of hypercholesterolemia, the diabetic rabbits showed a visible lipemia *in vitro* that was much more marked than that observed in the corresponding non-diabetic animals. The control and "alloxan-recovered" animals did not show more than a moderate to marked opalescence of the serum, while the diabetic animals exhibited a lipemia that was manifest as a milky or creamy appearance that rendered the serum opaque. A further difference in the lipemia of the diabetic and non-diabetic animals was the rapidity with which

TABLE I
Summary of Experimental Data

Series	No.	Sex	Experimental type*	Duration of cholesterol feeding	Total dose of cholesterol	Weight at beginning of cholesterol feeding	Weight at completion of cholesterol feeding	Average blood sugar	Average free serum cholesterol	Average total serum cholesterol	Lipemic index (0-4)	Grade of aorticath- ero- sclerosis (0-4)
				days	gm.	kg.	kg.	mg. per cent	mg. per cent	mg. per cent		
1	1	M	D	91	16	2.98	2.61	508	68	114	0	0
	2	M	D			2.12	1.89	583	89	111	0	0
	3	M	D			2.74	2.92	397	13	32	0	0
	4	M	C			2.56	3.20	109	11	30	0	0
	5	M	AR			3.08	3.51	108	14	32	0	0
2	6	M	D	90	31	2.53	2.24	501	51	71	0	0
	7	M	D			2.09	2.50	473	20	42	0	0
	8	M	C			3.26	4.66	116	11	23	0	0
	9	M	C			3.09	3.75	115	24	55	0	0
3	10	F	D	91	38 (dry)	2.45	1.98	517	18	53	0	0
	11	M	D			3.15	2.64	472	45	90	0.8	0
	12	F	C			3.54	4.06	96	25	84	0	1
	13	F	C			3.08	3.36	96	33	130	0	1
	14	M	C			2.84	2.76	100	27	103	0	1
4	15	M	D	52	39	2.23	2.05	329	220	726	2	0
	16	M	C			2.28	2.63	136	41	120	1	1
	17	M	C			2.41	2.07	153	414	1728	1.3	3
	18	M	AR			3.24	3.58	126	14	48	0	0
5	19	M	D	53	40	2.22	1.59	481	219	423	3.75	0
	20	M	D			2.37	1.88	427	101	197	2.75	0
	21	F	C			4.58	5.15	119	64	210	0.5	1
	22	F	C			3.50	4.59	131	105	316	1	1
	23	F	C			3.02	3.72	129	111	389	1.5	2
	24	M	AR			3.32	3.63	111	32	77	0.25	1
6	25	M	D	90	46	2.58	1.84	355	192	396	3.7	1
	26	F	D			2.73	2.60	354	57	159	1.7	1
	27	F	C			3.90	4.14	114	155	334	1.7	4
	28	F	C			3.62	4.40	123	139	349	2.0	4
	29	M	AR			3.04	3.23	116	104	359	1.8	4
	30	M	AR			3.43	3.78	108	89	232	1.2	4
	31	F	AR			3.55	3.98	115	100	367	1.8	4
7	32	M	D	89	48.5	3.48	3.45	452	99	277	2	1
	33	M	C			4.38	5.25	114	15	63	0	2
	34	M	C			3.40	3.68	118	66	167	1.4	3
	35	M	C			3.29	3.13	111	97	316	1.8	4
	36	M	AR			4.45	4.99	110	59	140	1	3
	37	M	AR			3.94	4.31	116	60	123	1	3

TABLE I—*Concluded*

Series	No.	Sex	Experimental type*	Duration of cholesterol feeding	Total dose cholesterol	Weight at beginning of cholesterol feeding	Weight at completion of cholesterol feeding	Average blood sugar	Average free serum cholesterol	Average total serum cholesterol	Lipemic Index (0-4)	Grade of aortic atherosclerosis (0-4)
				days	gm.	kg.	kg.	mg. per cent	mg. per cent	mg. per cent		
8	38	M	D	76	52	2.47	2.89	375	171	401	4	0
	39	M	C			3.50	4.06	113	121	425	1.75	4
	40	M	C			3.72	4.09	118	70	272	1.25	2
	41	M	AR			3.66	4.23	121	98	340	1.5	3
	42	M	AR			3.12	—	136	159	472	2.25	4
	43	M	MD			3.70	4.00	147	120	309	1.0	1
9	44	M	D	82	52	2.96	3.36	335	459	1970	1.6	2
	45	M	C			2.06	2.83	145	344	632	1.25	1
	46	M	C			2.49	4.10	137	179	334	0.75	3
	47	M	C			2.34	4.17	136	191	491	1.25	4
	48	M	AR			2.94	3.11	177	612	1849	1.5	4
	49	M	AR			3.57	4.50	127	75	357	0.8	1
10	50	M	D	82	60	3.07	3.38	444	505	1075	3.8	1
	51	M	D			3.10	2.19	398	315	703	3.2	1
	52	F	C			4.05	4.20	126	164	562	1.6	4
	53	F	C			3.73	4.51	117	192	602	1.6	2
	54	F	AR			3.85	5.09	115	235	656	2.0	3
11	55	F	D	89	65	2.69	3.31	392	135	346	1.2	1
	56	M	C			2.80	4.17	124	55	221	0	1
	57	M	C			3.17	4.36	128	123	450	1	3
	58	M	C			3.50	4.51	122	143	593	1.3	4

* D = diabetic; C = control; AR = "alloxan-recovered;" MD = mild diabetic.

this phenomenon became apparent after the blood was drawn. Lipemia was apparent in the blood of diabetic animals within 1 to 20 minutes. It was seldom seen in the blood of comparable non-diabetic animals before 30 to 60 minutes after the blood was drawn.

While the data considered above are consistent, the variability of the cholesterol dosage and of the duration of the experiments precludes a detailed analysis of the possible influence of certain other experimental variables, such as sex and change in weight of the animals during the experiment. It is interesting, therefore, to compare the data of individual animals that were similar in as many respects as possible, in order to assess the importance of these factors. The protocols of a number of such animals are given below.

The following protocols are of a diabetic rabbit and of a non-diabetic control

animal that are comparable as to sex and in all other respects, except that in the diabetic animal the degree of lipemia was much greater, and the amount of lipid

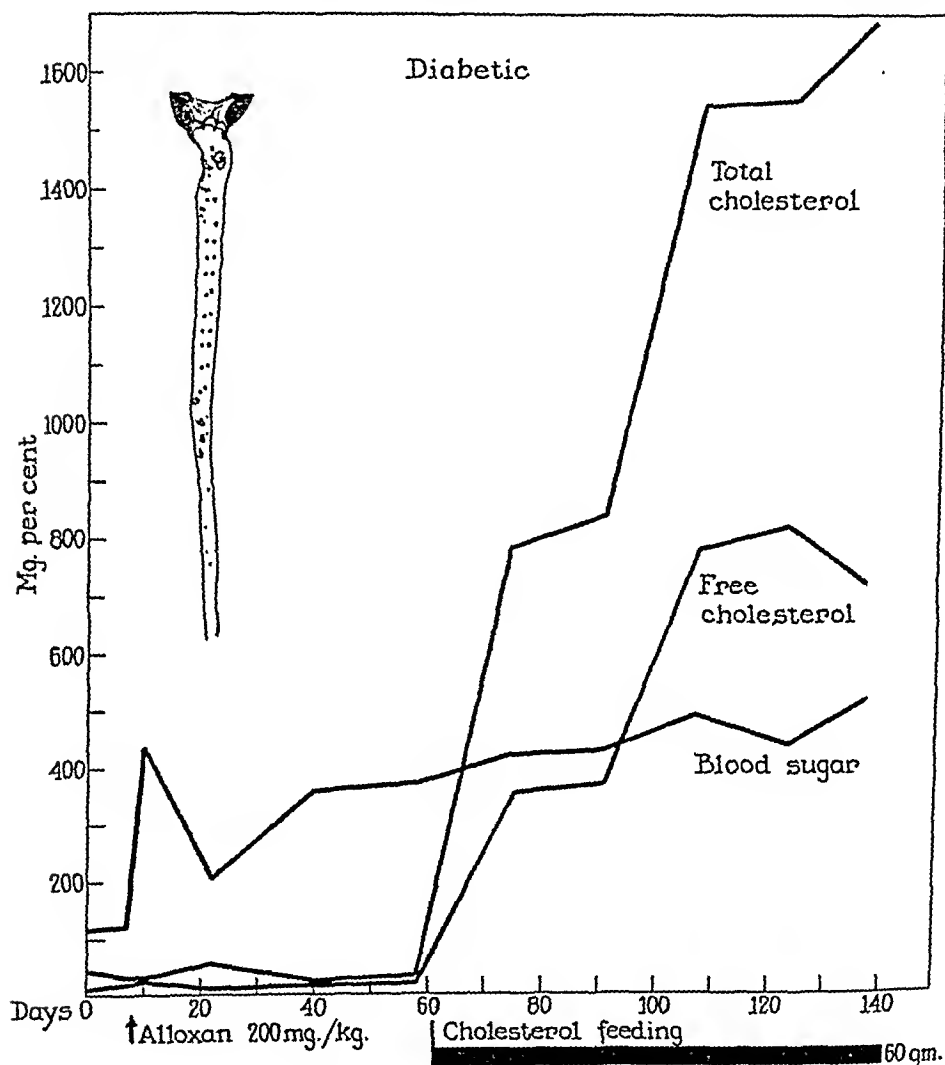


FIG. 2. Diagram and graph illustrating the procedures and findings in diabetic rabbit 50, series 10. The aortic atherosclerosis shown was recorded as grade 1.

deposition in the aorta, liver, spleen, and adrenal was much less than in the control rabbit.

No. 38. Diabetic. Male.—Dose of cholesterol per day, 0.68 gm. Duration of feeding, 76 days. Average total serum cholesterol, 401 mg. per cent. Lipemic index, 4.0. Ather-

osclerosis, grade 0. Liver fat, grade 0. Splenic fat, grade 1. Adrenal fat, normal. Weight gain, 0.42 kilo.

No. 39. Control. Male.—Dose of cholesterol per day, 0.68 gm. Duration of feeding, 76 days. Average total serum cholesterol, 425 mg. per cent. Lipemic index, 1.75. Ather-

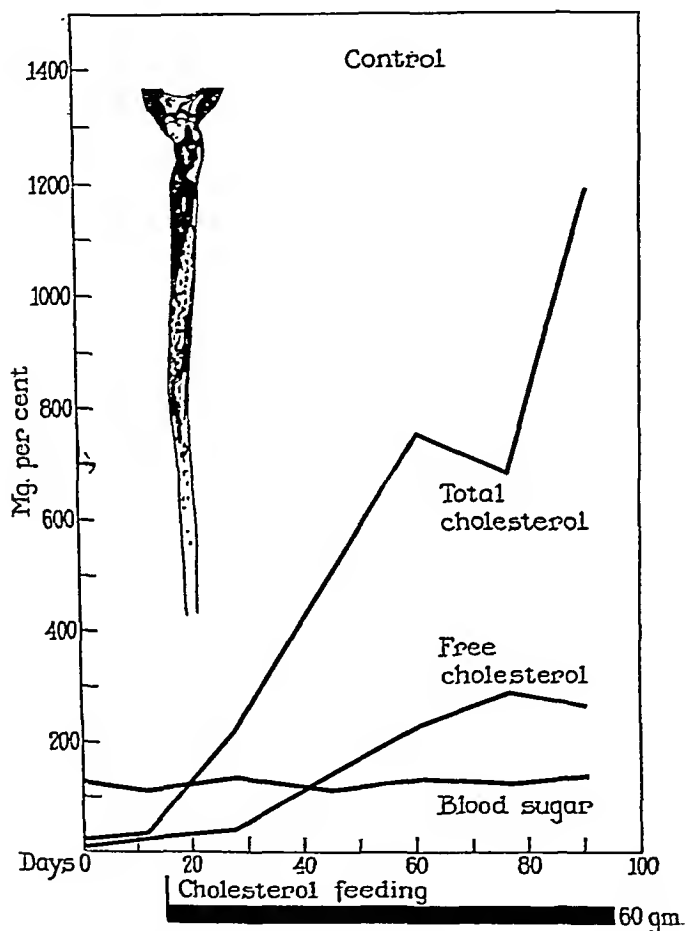


FIG. 3. Diagram and graph illustrating the procedures and findings in non-diabetic control rabbit 52, series 10. The aortic atherosclerosis shown was recorded as grade 4.

osclerosis, grade 4. Liver fat, grade 4. Splenic fat, grade 3. Adrenal fat, grade 4. Weight gain, 0.56 kilo.

The following two groups of protocols in which the comparable diabetic, "alloxan-recovered," and non-diabetic control animals alternate in sex, also indicate that sex is not the determining factor in inhibiting the deposition of lipids in the aorta and elsewhere.

No. 26. *Diabetic. Female*.—Dose of cholesterol per day, 0.52 gm. Duration of feeding, 90 days. Average total serum cholesterol, 159 mg. per cent. Lipemic index, 1.7. Atherosclerosis, grade 1. Liver fat, grade 1. Splenic fat, grade 1. Adrenal fat, grade 1. Weight loss, 0.13 kilo.

No. 36. "*Alloxan-Recovered*." *Male*.—Dose of cholesterol per day, 0.54 gm. Duration of feeding, 89 days. Average total serum cholesterol, 140 mg. per cent. Lipemic index, 1. Atherosclerosis, grade 3. Liver fat, grade 3. Splenic fat, grade 3. Adrenal fat, grade 3. Weight gain, 0.54 kilo.

No. 34. *Control. Male*.—Dose of cholesterol per day, 0.54 gm. Duration of feeding, 89 days. Average total serum cholesterol, 167 mg. per cent. Lipemic index, 1.4. Atherosclerosis, grade 3. Liver fat, grade 2. Splenic fat, grade 0. Adrenal fat, grade 2. Weight gain, 0.28 kilo.

No. 25. *Diabetic. Male*.—Dose of cholesterol per day, 0.52 gm. Duration of feeding, 90 days. Average total serum cholesterol, 396 mg. per cent. Lipemic index, 3.7. Atherosclerosis, grade 1. Liver fat, grade 1. Splenic fat, grade 1. Adrenal fat, normal. Weight loss, 0.74 kilo.

No. 29. "*Alloxan-Recovered*." *Male*.—Dose of cholesterol per day, 0.52 gm. Duration of feeding, 90 days. Average total serum cholesterol, 359 mg. per cent. Lipemic index, 1.8. Atherosclerosis, grade 4. Liver fat, grade 4. Splenic fat, grade 3. Adrenal fat, grade 4. Weight gain, 0.19 kilo.

No. 31. "*Alloxan-Recovered*." *Female*.—Dose of cholesterol per day, 0.52 gm. Duration of feeding, 90 days. Average total serum cholesterol, 367 mg. per cent. Lipemic index, 1.8. Atherosclerosis, grade 4. Liver fat, grade 1. Splenic fat, grade 0. Adrenal fat, grade 2. Weight gain, 0.43 kilo.

No. 27. *Control. Female*.—Dose of cholesterol per day, 0.52 gm. Duration of feeding, 90 days. Average total serum cholesterol, 334 mg. per cent. Lipemic index, 1.7. Atherosclerosis, grade 4. Liver fat, grade 2. Splenic fat, ? Adrenal fat, grade 2. Weight gain, 0.24 kilo.

No. 28. *Control. Female*.—Dose of cholesterol per day, 0.52 gm. Duration of feeding, 90 days. Average total serum cholesterol, 349 mg. per cent. Lipemic index, 2. Atherosclerosis, grade 4. Liver fat, grade 1. Splenic fat, grade 1. Adrenal fat, grade 2. Weight gain, 0.78 kilo.

Inasmuch as equal daily doses of cholesterol were fed to rabbits of different body weights, it is conceivable that the apparent inhibitory effect of the diabetic state might have occurred because of the chance feeding to the non-diabetic animals of a higher dose of cholesterol per kilo of body weight. In the following protocols the diabetic and non-diabetic animals are matched as to sex, duration of cholesterol feeding, average total serum cholesterol, and, in addition, as to dosage of cholesterol calculated as an average dose per day per kilo of average body weight during the feeding period. It is apparent from examination of these protocols that the experimental result was not determined by actual or relative differences in the dosages of cholesterol.

No. 11. *Diabetic. Male*.—Dose of cholesterol (dry) per day per kilo, 145 mg. Duration of feeding, 91 days. Average total serum cholesterol, 90 mg. per cent. Lipemic index, 0.8. Atherosclerosis, grade 0. Liver fat, grade 0. Splenic fat, grade 1. Adrenal fat, normal. Weight loss, 0.51 kilo.

No. 14. *Control. Male*.—Dose of cholesterol (dry) per day per kilo, 150 mg. Duration

of feeding, 91 days. Average total serum cholesterol, 103 mg. per cent. Lipemic index, 0. Atherosclerosis, grade 1. Liver fat, grade 0. Splenic fat, grade 0. Adrenal fat, normal. Weight loss, 0.08 kilo.

No. 44. Diabetic. Male.—Dose of cholesterol per day per kilo, 203 mg. Duration of feeding, 82 days. Average total serum cholesterol, 1970 mg. per cent. Lipemic index, 1.6. Atherosclerosis, grade 2. Liver fat, grade 2. Splenic fat, grade 3. Adrenal fat, grade 2. Weight gain, 0.40 kilo.

No. 48. "Alloxan-Recovered." Male.—Dose of cholesterol per day per kilo, 201 mg. Duration of feeding, 82 days. Average total serum cholesterol, 1849 mg. per cent. Lipemic index, 1.5. Atherosclerosis, grade 4. Liver fat, grade 4. Splenic fat, grade 3. Adrenal fat, grade 4. Weight gain, 0.07 kilo.

Inspection of the data presented in all the groups of protocols given above indicates further that gain or loss of body weight during the course of the feeding period exercised no determining influence on the result of the experiment.

It may be added that it was not possible to select pairs or groups of diabetic and non-diabetic animals matched as in the groups detailed above that yielded evidence contrary to that already presented.

Careful morphological examination, both grossly and microscopically, of the aorta and other organs revealed in varying degrees, as already indicated in Table I, the lesions that have been described by many authors as the characteristic sequelae of prolonged cholesterol feeding in rabbits (2-4). Not only were these lesions characteristic in form and location in the cholesterol-fed control animals, but also in the "alloxan-recovered" and diabetic rabbits. The only distinguishable difference in the morphology of the aortic lesions was a quantitative one as detailed above. This was true also of the lipid deposition in the liver, spleen, and adrenal glands. It should be noted, moreover, that in those parts of the aorta and other arteries that were uninvolved by cholesterol atherosclerosis, no differences could be distinguished microscopically between the diabetic and non-diabetic rabbits. The diabetic animals regularly presented the lesion of Armanni in the kidney and the hydropic changes in the pancreatic islets and ductules that have been described in detail elsewhere as characteristic of prolonged alloxan diabetes (8). The islets of Langerhans and the pancreatic ductules in the "alloxan-recovered" animals lacked hydropic changes but careful study of the islets revealed a peculiar and characteristic disturbance of cell arrangement which was distinctly different from the normal. However, in no other organ, including the thyroid gland, was there any evidence of a histologic difference between the diabetic, "alloxan-recovered," and control animals.

II. THE EFFECT OF ALLOXAN DIABETES ON THE RETROGRESSION OF EXPERIMENTAL CHOLESTEROL ATHEROSCLEROSIS

Materials and Methods

Eighty-one young, adult, domestic white rabbits of both sexes were employed. Of these 32 completed a satisfactory experimental course, and comprised two experimental groups.

Each of the 25 rabbits in one group was fed 61.5 to 62.5 gm. of cholesterol during 88 to 90 days. In a second group of 7 rabbits each was fed 40.5 gm. of cholesterol during 59 days. The individual daily dose of cholesterol was 0.75 gm. fed by means of a stomach tube as a 5 per cent solution in warm corn oil.

Determinations at intervals of approximately 3 weeks were made of the non-fasting blood sugar, and of the free and total cholesterol content of the serum by a modified Folin micro method and the Schoenheimer-Sperry method respectively.

Immediately following the completion of the period of cholesterol feeding about one-half of the animals of each group received an intravenous injection of 150 mg. of alloxan per kilo of body weight administered as a 5 per cent aqueous solution. No insulin or other therapy was employed. The animals were given an unlimited diet of Purina rabbit chow and water *ad libitum*.

The animals, including diabetic, control, and those that recovered from the effects of a diabetogenic dose of alloxan after a brief period of diabetes ("alloxan-recovered"), were sacrificed at intervals of 1 to 16 weeks after the cessation of cholesterol feeding. Complete autopsies were performed and the tissues were treated in the same manner as described in Part I of this paper. The criteria of the diabetic state and the grading of the degree of aortic atherosclerosis and of the amount of lipid deposited in the liver, spleen, and adrenal were also the same as those used in the experiment described in Part I.

Observations

The experimental procedures and observations are summarized in Table II, in which the average values of serum cholesterol given are those obtained during the cholesterol feeding period, including one determination made the day before cholesterol feeding was begun. The blood sugar values are averages obtained after the cessation of cholesterol feeding. Among the animals that were injected with alloxan, the average of the blood sugar content includes one determination made before alloxan was administered. The period of retrogression was that between the cessation of cholesterol feeding and death of the animal. In the diabetic animals the duration of diabetes was the same as the period of retrogression.

The results as shown in Table II did not demonstrate that alloxan diabetes has any effect on the rate or degree of retrogression of experimental cholesterol atherosclerosis in the rabbit as judged by gross morphological examination of the aorta stained *in toto* with Sudan IV. Neither did microscopic examination of the entire length of the aorta reveal any appreciable differences between the atherosclerotic lesions of comparable diabetic and non-diabetic animals. The diabetic state was not found to alter appreciably the rate or degree of disappearance of abnormal lipid deposits in the liver, spleen, and adrenal cortex as judged by microscopic examination of appropriately stained frozen sections. In spite of the fact that in certain of the diabetic animals there occurred shortly after the administration of alloxan a distinct increase in the previously existing hypercholesterolemia, they in common with the other diabetic animals, the control, and "alloxan-recovered" rabbits, showed a return of the blood cholesterol to normal levels within 42 days. The only consistent biochemical differ-

TABLE II
Summary of Experimental Data

Series	No.	Sex	Experimental Type*	Total dose of cholesterol	Time of retrogression	Average blood sugar during retrogression	Average free serum cholesterol	Average total serum cholesterol	Aortic atherosclerosis (0-4)
				gm.	wks.	mg. per cent	mg. per cent	mg. per cent	
1	1	F	D	61.5	1	—	90	277	1
	2	F	D	61.5		570	134	382	4
	3	F	C	61.5		122	174	398	3
2	4	F	D	61.5	3	357	163	471	3
3	5	F	D	61.5	5	384	144	426	2
	6	F	C	61.5		134	161	516	4
	7	F	C	61.5		148	107	291	3
4	8	F	D	61.5	6	319	194	472	4
5	9	F	D	61.5	7	425	96	307	3
	10	M	C	61.5		121	—	—	1
6	11	F	D	62.25	9	353	106	261	2
	12	F	C	61.5		121	140	440	4
7	13	F	MD	61.5	11	286	135	430	3
	14	M	D	61.5		410	218	525	4
	15	M	AR	61.5		138	124	318	2
	16	M	AR	61.5		175	81	208	4
	17	M	AR	61.5		163	163	487	2
8	18	F	D	61.5	16	396	183	479	2
	19	F	D	61.5		488	232	480	2
	20	F	C	61.5		133	—	—	2
	21	M	C	61.5		126	83	298	4
	22	M	C	61.5		120	126	371	4
	23	M	C	61.5		111	88	325	2
	24	F	C	61.5		124	143	382	4
	25	F	C	61.5		127	72	240	3
9	26	F	D	40.5	6	309	140	320	3
	27	F	D	40.5		373	88	262	3
	28	F	C	40.5		130	138	424	4
	29	M	C	40.5		120	41	187	3
	30	F	C	40.5		128	145	452	3
	31	F	C	40.5		125	71	214	2
	32	M	AR	40.5		124	83	270	4

* D = diabetic; C = control; AR = "alloxan-recovered;" MD = mild diabetic.

ence observed between the various groups of rabbits was the hyperglycemic state of the diabetic animals. The only morphological differences found to exist between diabetic and non-diabetic animals were the changes in the pancreas and kidney that are associated with the injection of alloxan or with the development of persistent diabetes.

In both the diabetic and non-diabetic animals there was no gross morphological evidence that any appreciable retrogression of the aortic atherosclerosis had occurred during periods of up to 4 months' duration. However, in animals that survived a period of retrogression of 6 weeks or more there was definite microscopic evidence of the disappearance of lipid deposits from the aorta, splenic arterioles, and other small arteries, from the reticulo-endothelial cells of the liver and spleen, and from the parenchymal cells of the liver and adrenal cortex. In the aorta, the gradual disappearance of lipid material was accompanied by a gradual increase in the number of fibroblastic cells in the atherosclerotic lesions.

DISCUSSION

The observations recorded in Part I of this paper demonstrate clearly that, under the conditions described, there was associated with the presence of alloxan diabetes a marked but incomplete inhibition of the development of experimental cholesterol atherosclerosis in cholesterol-fed rabbits. There was also an inhibition of the deposit of sudanophilic lipid substances in the reticulo-endothelial cells of the liver and spleen and in the parenchymal cells of the liver and adrenal cortex. This inhibition occurred in spite of the induction of a marked degree of hypercholesterolemia in many of the diabetic animals, hypercholesterolemia that was usually as high as, and often higher than, that observed in the corresponding control animals.

This inhibitory effect was apparently dependent neither upon the administration of alloxan *per se* nor upon the short initial period of insulin and dextrose therapy, inasmuch as both the diabetic and "alloxan-recovered" animals received such injections before cholesterol feeding was instituted, but only in the diabetic rabbits was the inhibitory effect apparent. The "alloxan-recovered" animals responded to cholesterol feeding exactly as did the control animals. In addition, it was found that the inhibitory effect was not dependent on the sex or weight of the animal, nor upon the daily dosage of cholesterol, the form in which it was administered, nor the duration of cholesterol feeding. The effect was also independent of changes in body weight occurring during the course of our experiments and of the actual degree of hypercholesterolemia induced by the administration of cholesterol. Moreover, there was no gross or histological evidence of a morphological basis for the inhibitory effect either in the aorta or in the other organs in which it was observed. Indeed, the only observed factors consistently associated with the inhibition of the expected morphological effects of cholesterol feeding were the diabetic state and a degree

of visible lipemia considerably greater than that observed in the control animals. A moderate degree of visible lipemia in the control animals was regularly associated with the development of severe atherosclerosis of the aorta. On the contrary, a marked degree of visible lipemia was observed in a large proportion of the diabetic animals that presented at autopsy only a minimal degree of aortic atherosclerosis.

Objection might possibly be raised to our inference that the injection of alloxan *per se*, apart from its diabetogenic effects, was not responsible for the inhibitory effect observed in the diabetic animals on the ground that the "alloxan-recovered" rabbits did not provide a valid control of this possible factor. It could be argued that the "alloxan-recovered" animals were less susceptible to the general effects of alloxan than the diabetic animals as indicated by the very fact that permanent diabetes failed to develop in them. Animals that fail to respond with the development of permanent diabetes to a dose of alloxan that is diabetogenic to a majority of the species are frequently referred to in the literature rather loosely as "alloxan-resistant." However, in our "alloxan-recovered" animals, the diabetogenic effect of alloxan was manifested initially in the production of a temporary diabetic state of mild or moderate severity from which spontaneous recovery occurred within the period of several weeks before cholesterol feeding was started. Clearly, the injection of alloxan was effective, at least to a degree, but these particular rabbits displayed a capacity for recovery that distinguished them from the permanently diabetic animals.

Although we have referred to these animals as "alloxan-recovered" there is reason to believe that such animals have not returned to a strictly normal state. In our present observations, the "alloxan-recovered" animals during the period of cholesterol feeding showed neither obvious nor chemical evidence of diabetes. However, in other experiments (9) more detailed and precise studies have demonstrated that alimentary glycosuria may be present in such animals in spite of normal fasting blood sugar levels and that this is dependent on the occurrence of slight postprandial hyperglycemia. These residual metabolic defects are correlated with the presence of definite histological alterations in the islets of Langerhans which were detectable in the animals of our present experiments months after the injection of alloxan.

There is ample evidence, therefore, to indicate that our "alloxan-recovered" animals did not tolerate the injection of alloxan without suffering from its effects. Accordingly, we are inclined to regard these animals, at least tentatively, as providing a suitable control of the effects of alloxan injection *per se*. Further experiments currently in progress are designed to settle this point definitely by determining whether the inhibitory effect observed in alloxan-diabetic rabbits is abolished by controlling the diabetic state with insulin.

While we are quite unable to offer a specific explanation of the inhibitory

effect observed in the present experiments, it would appear, in view of the considerations set forth in the preceding paragraphs, that it is dependent upon some undefined factor or factors implicit in, or closely associated with, the diabetic state. That the diabetic condition or factors associated with it exercised an influence on the state and stability of the blood lipids in rabbits fed cholesterol in oil is indicated not only by the development of a marked visible lipemia, but also by the inhibition of lipid deposition in the aorta and elsewhere. Since the deposition of lipids in the intima of arteries is an essential feature of the development of experimental cholesterol atherosclerosis, particular interest attaches to any evidence of an alteration of the physicochemical state of the lipids in the blood plasma (*viz.* excessive lipemia) that coexists with protection from the usual effects on the arteries of rabbits associated with hypercholesterolemia.¹

Almost from the first demonstration of the fact that the feeding of cholesterol is capable of producing atherosclerosis in the arteries of rabbits (10), it was recognized that the development of the arterial lesions is associated with a significant elevation of the cholesterol content of the blood (11). This was confirmed repeatedly by subsequent studies which showed that, in general, the severity of the induced atherosclerosis is correlated with the degree and duration of the induced state of hypercholesterolemia (2-4). It has also been shown that the development of experimental cholesterol atherosclerosis can be inhibited by various modifications of the experimental procedure that prevent the development of the expected degree of hypercholesterolemia (3, 4). On the basis of such data, the concept arose that hypercholesterolemia is the sole factor of importance in the genesis of experimental atherosclerosis. This concept was seriously questioned some years ago by Duff (3) on the basis of the evidence then available and, more recently, certain other investigators have emphasized that factors other than hypercholesterolemia may be important in the development of experimental atherosclerosis, pointing out that animals with comparable levels of induced hypercholesterolemia frequently exhibit widely differing degrees of atherosclerosis (12-15).

The absolute inhibition of the development of experimental cholesterol atherosclerosis in an appreciable number of the diabetic rabbits in our experiments in spite of the presence of marked and prolonged hypercholesterolemia shows clearly that the mere existence of a markedly increased quantity of cholesterol in the circulating blood for a considerable length of time is not in itself capable of causing lesions in the arteries. This conclusion is supported by the demonstration of Steiner (16, 17) and others (18, 19) that the addition of choline to the diet inhibits the development of atherosclerosis in cholesterol-fed

¹ This evidence clearly conflicts with the hypothesis of Moreton (*Science*, 1947, 106, 190; and 1948, 107, 371) which postulates that the determining factor in the development of atherosclerosis is the presence in the circulating blood of lipid particles of large size such as are present in abundance in the grossly milky or creamy serum of hyperlipemic states.

rabbits, though it does not prevent the development of marked hypercholesterolemia (16, 17). It is evident, therefore, that the development of experimental cholesterol atherosclerosis is dependent not only upon the occurrence of hypercholesterolemia *per se* but also upon another essential factor or factors as yet undetermined.

We are fully aware that our experimental observations are at variance with the evidence adduced to show that diabetes mellitus in man promotes the development of arteriosclerosis. A logical resolution of this apparent conflict compels consideration of one or more of the following possibilities. First, the conflict may be consequent on species differences. Second, alloxan diabetes in the rabbit may not be metabolically comparable with diabetes mellitus in man. Third, experimental cholesterol atherosclerosis in rabbits may not be comparable with the type of arterial disease to which diabetic patients are prone. Fourth, the impression that occlusive arterial disease in diabetic patients is dependent upon an excessive development of atherosclerosis of the intima of arteries may be erroneous. Obviously, it is impossible in the present state of knowledge to predict which of the possibilities just mentioned may prove to be correct.

The experimental data presented in Part II of this paper clearly fail to demonstrate any difference in the rate of retrogression of experimental cholesterol atherosclerosis in alloxan-diabetic rabbits as compared with control animals. In our experiments the period of retrogression, *i.e.* the period after the feeding of cholesterol was terminated, was limited to 4 months. Whether experiments with longer periods of retrogression would show any differential effect is impossible to say. More prolonged experiments, however, would be technically difficult because of the excessive mortality from alloxan diabetes of long duration in rabbits.

Our negative results are similar to those reported by other investigators who have attempted to influence the retrogression of experimental cholesterol atherosclerosis by the administration of potassium iodide (20, 21). On the other hand, Steiner (22) some years ago brought forward highly suggestive evidence of the ability of choline to bring about some reabsorption of arterial lesions previously induced by cholesterol feeding. Added to this evidence is the recent report of Morrison and Rossi (23) who have described complete reabsorption of the lesions of experimental cholesterol atherosclerosis in 17 out of 23 rabbits given larger daily doses of choline over a period of 182 days after the cessation of cholesterol feeding.

The observations, already cited relative to the effects of choline on the development and retrogression of experimental cholesterol atherosclerosis, coupled with the results of our own experiments on the effects associated with the presence of alloxan diabetes, permit of interesting deductions regarding the process of lipid accumulation in the walls of the arteries of cholesterol-fed rabbits. If

all the observations are correct and correctly interpreted, it is evident that the process of lipid accumulation must represent the resultant of the effects of two separate and distinct sets of factors, those factors the balance of which hinders or promotes the deposit of lipids in the arterial walls, and another set of factors the balance of which hinders or promotes their removal after they are deposited. This is not a new concept but the means of demonstrating its correctness have not hitherto been available. Since alloxan diabetes (or some associated influence) inhibits the development of experimental cholesterol atherosclerosis but has no noticeable effect on the retrogression of the arterial lesions, it follows that the inhibitory effect must be implemented solely or almost solely by interference with the deposit of lipids. On the other hand, the results of Morrison and Rossi (23) indicate that the administration of choline has a powerful effect in facilitating the removal of lipids already deposited in the arterial wall. The effect of choline in inhibiting the development of experimental cholesterol atherosclerosis, as described by Steiner (17) could, therefore, be due either to interference with the deposition of lipids, or to facilitation of their removal as rapidly as they are deposited or to a combination of both effects. However, if choline facilitates the removal of lipids already deposited in the arterial walls, this is the only effect of choline on experimental atherosclerosis that is susceptible of proof by the types of experiment under consideration here. The mechanisms of interference with the deposit of lipids and of facilitation of their removal remain to be investigated and clarified but the means to do so appear now to be at hand.

It is evident that our experimental results find no direct application to the problem of arterial disease in human diabetes. Nevertheless, if it is true that the accumulation of lipids in the intima of arteries is a central feature of human atherosclerosis, as it appears to be of experimental cholesterol atherosclerosis, then the isolation of the factors governing this fundamental biological process in the experimental animal may be expected to help in elucidating the nature of the same process in man.

SUMMARY

A comparison was made of the effects of cholesterol feeding in normal rabbits and in rabbits rendered persistently diabetic by means of alloxan. In the two groups of animals hypercholesterolemia of comparable degree was induced by the feeding procedure. Nevertheless, the severity of the atherosclerosis of the aorta produced in the diabetic rabbits was much less than in the non-diabetic control animals. Indeed, a large proportion of the diabetic animals presented no atherosclerosis whatever. There was a similar inhibition of the deposit of lipid substances in the liver, spleen, and adrenal glands of the diabetic rabbits.

The inhibition of the development of experimental cholesterol atherosclerosis which was associated with the presence of alloxan diabetes was independent of

the administration of alloxan *per se*. It was not dependent on the sex or weight of the animal, nor upon the daily dosage of cholesterol, the form in which it was administered, nor the duration of cholesterol feeding. It was also independent of changes in body weight occurring during the course of our experiments and of the actual degree of hypercholesterolemia induced by the administration of cholesterol. In addition, there was no gross or histological evidence of a morphological basis for the inhibitory effect either in the aorta or in the other organs in which it was observed.

Only two factors were observed to be consistently associated with the inhibition of the expected morphological effects of cholesterol feeding, namely, the diabetic state and a degree of visible lipemia considerably greater than that observed in the control animals.

The inhibitory effect observed in these experiments would appear to depend upon some as yet undetermined factor or factors implicit in the diabetic state or closely associated with it. The experimental data presented demonstrate clearly that hypercholesterolemia is not the sole factor concerned in the genesis of experimental cholesterol atherosclerosis, but that another factor, or factors, rendered inoperative in our experiments must be essential to the production of the arterial lesions.

In view of the inhibitory effect on the development of experimental cholesterol atherosclerosis observed in alloxan-diabetic rabbits, the effect of alloxan diabetes on the retrogression of such arterial lesions was studied in another series of experiments. No effect on retrogression could be demonstrated within periods lasting up to a maximum of 4 months after the cessation of cholesterol feeding.

The results of our two series of experiments, considered together, indicate that the process of deposition of lipids in the arterial walls is governed by factors different from those that are operative in the process of removal of lipids after they have been deposited. The inhibition of the development of experimental cholesterol atherosclerosis in alloxan-diabetic rabbits must depend on interference with the process of deposition of lipids and not on a process of removal of lipids as fast as they are deposited.

Our experimental results find no direct application to the problem of arterial disease in human diabetes. Nevertheless, the experimental procedures that we have employed provide a new basis for the design of further experiments directed toward the elucidation of the nature of the unknown factors that govern the process of lipid deposition in the walls of arteries.

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be demonstrated by studying these two factors not only as they progressed following DCA administration, but also as they regressed following withdrawal of DCA.

One hundred and ten albino rats of the Sherman strain, averaging approximately 90 gm. in weight were divided into two groups. Forty-one animals served as untreated controls while the remaining 69 received DCA with 1 per cent saline as drinking water *ad libitum*. DCA was administered as one-third of a 75 mg. pellet (Schering cortate) subcutaneously implanted on the 1st day of the experiment and every 2 weeks thereafter. During the 4th week of the experiment renal function (7) and blood pressure (8) were determined in a group of 9 of the control and 18 of the DCA-saline-treated animals. Immediately following these procedures the control group and half of the treated group were sacrificed, their kidneys fixed, and then weighed. Pellets were removed from the remaining half of the DCA-saline-treated group which were then returned to tap water.

During the 6th week of the experiment renal function and blood pressure were again determined in a group of 8 control and 20 of the DCA-saline-treated animals, as well as in the group whose DCA-saline treatment had stopped 2 weeks earlier. Again, all animals were sacrificed except for half of the DCA-saline-treated group in which treatment was stopped at this time.

During the 8th week, the usual procedures were carried out in a control group, in a DCA-saline-treated group, and in the group whose treatment had been stopped in the previous period. Insufficient DCA-saline-treated animals remained to permit the experiment to be carried out beyond this period. Consequently, a subsidiary experiment was set up to determine the effects of stopping treatment in the 8th week. The base line data for control and DCA-saline-treated groups in the 8th week were again determined. Treatment was stopped in half of the treated group and their renal function and blood pressure determined again 1 week later.

Briefly, the clearance technique was as follows: PAH solution (12.5 mg./cc. in 2 per cent sodium sulfate) is injected into the lumbar region in accordance with a dose-body weight scale calculated to yield the desired plasma level of 5 to 7 mg. per cent at 50 minutes. Immediately following this injection, 3 cc. of warm 2 per cent inulin solution is injected intraperitoneally. The completion of this second injection marks the start of the urine collection period, and the rat is immediately placed into a metabolism funnel.

Fifty minutes after injection, the rat is picked up over the funnel and the bladder drained by suprapubic pressure, although micturition is usually free and spontaneous. Immediately following urine collection, 0.75 cc. of blood is obtained by heart puncture. Plasma and urine are then analyzed for inulin and PAH.

Blood pressure determinations are made with the tail plethysmograph using ether as anesthetic. While this method may fail to record a raised pressure in the occasional animal which is actually hypertensive, experience in many hundreds of animals indicates that it gives reliable and reproducible results. The actual pressure recorded is somewhat below the systolic level.

The results obtained in the two separate parts of the experiment (up to the 8th week, and then beyond this period) are presented in Table I. The number of animals indicated in each case refers to those sacrificed in that particular period. For simplicity, and in order to clarify the significant findings, the data have been rearranged graphically in Fig. 1. In this figure, the findings are presented as percentage deviation of the test group values from those obtained in a group of intact controls studied on the same day. Changes which are statistically significant ($p < 0.02$) are denoted.

TABLE I

Blood Pressure, Renal Function, and Kidney Weight Determined in Groups of Sherman Rats at Various Stages of Treatment with DCA and Saline, and Subsequent to the Cessation of Treatment

	Control	DCA-saline	DCA-saline treatment stopped in previous period
No. of animals.....	9	9	
Blood pressure.....	106 ±12	131 ±16	
CIN, cc./100 cm. ²	0.34 ±0.03	0.34 ±0.03	
4th wk. CPAH, cc./100 cm. ²	2.48 ±0.33	2.70 ±0.45	
TmpAH, mg./100 cm. ²	0.125 ±0.014	0.121 ±0.021	
FF as per cent.....	13.7	12.9	
CPAH/TmpAH.....	19.8	22.2	
Kidney weight, mg./100 cm. ²	397 ±42	554 ±44	
No. of animals.....	8	10	9
Blood pressure.....	111 ±16	134 ±16	106 ±10
CIN, cc./100 cm. ²	0.31 ±0.07	0.32 ±0.09	0.34 ±0.03
6th wk. CPAH, cc./100 cm. ²	2.47 ±0.27	2.81 ±0.76	2.73 ±0.37
TmpAH, mg./100 cm. ²	0.127 ±0.010	0.134 ±0.033	0.129 ±0.011
FF as per cent.....	12.5	11.4	12.4
CPAH/TmpAH.....	19.4	20.9	21.2
Kidney weight, mg./100 cm. ²	439 ±49	520 ±93	507 ±30
No. of animals.....	8	10	10
Blood pressure.....	107 ±13	151	97 ±17
CIN, cc./100 cm. ²	0.33 ±0.03	Not graphed	0.39 ±0.03
8th wk. CPAH, cc./100 cm. ²	2.59 ±0.27		2.87 ±0.39
TmpAH, mg./100 cm. ²	0.120 ±0.017		0.131 ±0.015
FF as per cent.....	12.7		13.7
CPAH/TmpAH.....	21.7		21.9
Kidney weight, mg./100 cm. ²	459 ±45	602	501 ±39

ment either at 25, 37, or 51 days, blood pressure fell promptly to normal levels. Indeed, 6 days after cessation of DCA treatment which had been carried on for 51 days, a significant elevation of pressure was no longer demonstrable. Since blood pressure determinations were not carried out at times other than those specified in the table, the return of the elevated pressure to normotensive levels may have occurred even more rapidly than is here indicated.

Renal Function.—Renal function appeared undisturbed after 25 days and after 37 days of treatment with DCA-saline. This is in accord with our previous finding that Sherman animals do not develop renal functional changes as readily as do Wistar (9). Fifty-one days of treatment resulted in a significant increase in the glomerular filtration rate, and consequently, in the filtration fraction. Since in the two earlier periods renal function as here determined was unaffected by the DCA-saline treatment, it is not surprising that no real change was observed 12 days after cessation of treatment in either case. The marked deviation in filtration rate observed after 51 days of treatment disappeared 6 days after treatment was stopped.

The data concerning renal function fall in the same direction as others which we have reported and would, alone, suggest that the elevation in blood pressure is independent of renal functional derangement. Further, since change in renal function may only be observed relatively late in the course of treatment, the idea that it results from the elevated pressure might well be entertained. This suggestion is, however, contradicted by the observations concerning renal weight.

Kidney Weight.—Bearing in mind the obvious fact that changes in renal mass can probably not occur as rapidly as alterations in blood pressure, a remarkable parallel between these functions was observed. Elevation of the blood pressure even at the earliest date studied was accompanied by an increase in kidney weight, while restoration of the blood pressure was accompanied by a return towards normal of kidney weight. Since renal function was maintained only at the normal level despite this increase in size, it seems reasonable to assume that this process is a compensatory hypertrophy.

It is of some interest in relation to the theory of renal function tests that this renal involvement becomes apparent at once when renal function is related to actual renal mass.

The apparent correlation between renal mass and blood pressure was subjected to statistical analysis. In Fig. 2, renal mass in the DCA-saline-treated animals is plotted as a frequency distribution against blood pressure, the data being taken from part one of the experiment. The regression line for the data in which b , the coefficient of regression is 0.124, is statistically significant. Since this graph represents all treated animals, whether or not pellets were removed, the significant regression assumes even greater meaning. The

conclusion that the kidneys are in some way involved in DCA hypertension from the beginning of the process appears unavoidable.

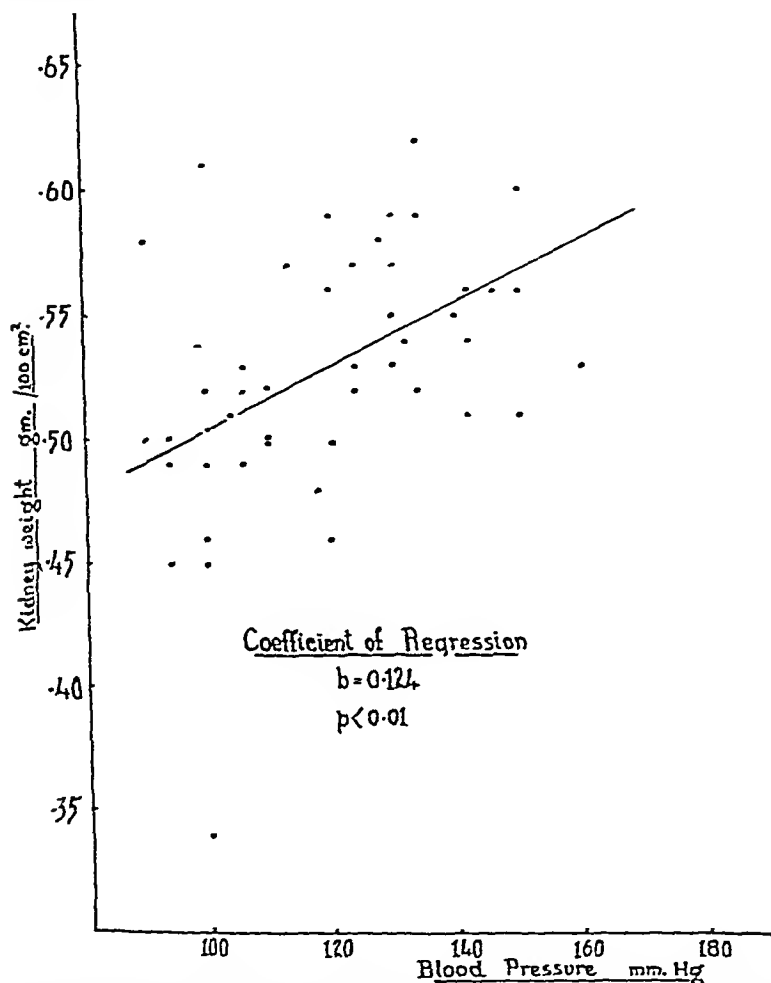


FIG. 2. Renal mass in DCA-saline-treated animals plotted as a frequency distribution against blood pressure.

Experiment 2

Since the kidneys are thus apparently immediately involved following DCA administration, two major possibilities concerning the mechanism suggest themselves—(a) the kidneys liberate a pressor substance upon stimulation by DCA or (b) the kidneys are actively concerned in the excretion and possible inactivation of DCA.

It seemed to us that the first step in distinguishing between these possibilities would be to examine the effects of DCA on blood pressure in the absence of the kidneys. The basic principle followed was to administer DCA until the blood pressure had attained a predetermined level, and then to nephrectomize the animals. A fall in pressure following nephrectomy would offer strong support to the idea that the pressor effect of DCA had been mediated by the kidney, perhaps through stimulation of a renal pressor mechanism. On the other hand, a rise in the blood pressure following nephrectomy would indicate that the pressor action of DCA was a more direct phenomenon.

Four separate experiments were carried out. In two of these, the blood pressure was elevated to a significant but low degree before nephrectomy so that either a fall or a rise might be easily discerned. Two were performed at an earlier stage in DCA treatment when the blood pressure was not yet significantly elevated. Since all four experiments yielded the same fundamental result only one experiment of each group is here reported.

Experiment 2a.—Twenty-eight male albino rats of an inbred Wistar strain and approximately 150 gm. in weight were maintained for 29 days. Eight animals served as untreated controls while the remaining 20 received a DCA pellet (one-third of a 75 mg. cortate pellet) as a subcutaneous implant on the 1st and 14th days of the experiment. On the 20th day, the left kidney was removed from each of 12 of the DCA-treated animals and on the 27th day of the experiment the remaining kidneys were removed. Blood pressure was determined at 1 or 2 day intervals beginning on the 18th day. The findings for this experiment are presented in Fig. 3. Seven of the 12 nephrectomized animals survived for the blood pressure determination 24 hours after complete nephrectomy, 2 for the 48 hour period.

Blood pressure was significantly elevated in the DCA-treated groups at the time of the first blood pressure determination on the 18th day. A fall in blood pressure occurred immediately following removal of one kidney but this was only temporary. In contrast, 24 hours after removal of the second kidney, a significant elevation above both untreated and DCA-treated controls was observed in the blood pressure of the nephrectomized animals. This result is the more remarkable since it occurred despite the undoubted operative shock, a factor not present in the control groups, and was observed not only as a group average but also in 6 of the 7 survivors, while the pressure of the seventh animal did not fall. The further elevation observed at 48 hours cannot be considered since it is based on only two survivors.

Experiment 2b.—Thirty male albino rats of an inbred Wistar strain, approximately 150 gm. in weight, were maintained for 14 days. Fifteen of these animals received 2 pellets (one-third of a cortate pellet) on the 1st day of the experiment and a third pellet on the 6th day. On the 12th day, 9 control and 9 DCA-treated animals were subjected to a one stage bilateral nephrectomy. Blood pressure was determined daily beginning on the 11th day. The results are presented in Fig. 4. All animals survived in good shape for 24 hours, but only 4 of the DCA-treated and 1 of the untreated nephrectomized animals were available for the 48 hour determination.

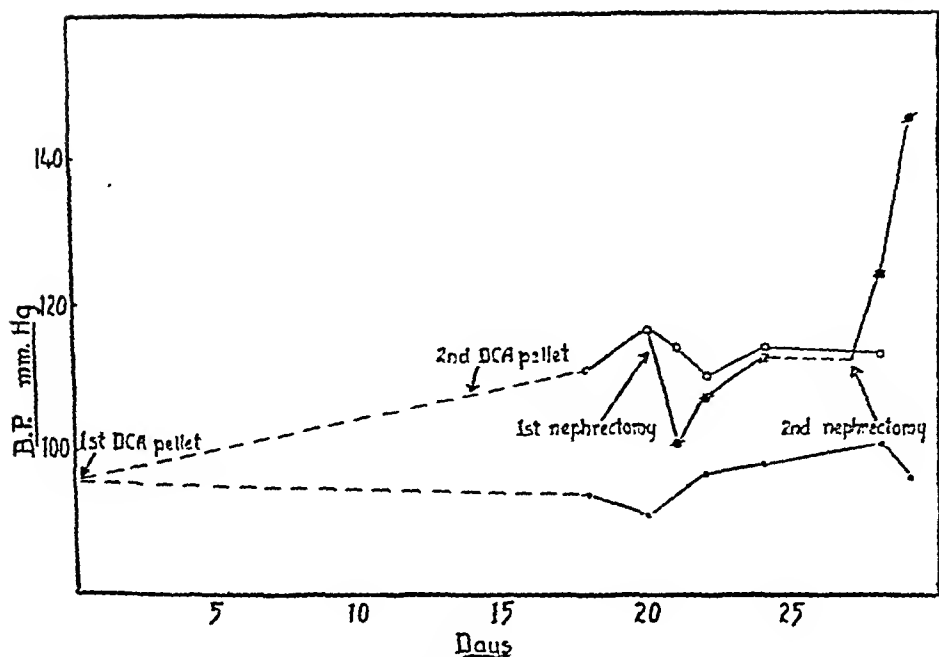


FIG. 3. The blood pressure in DCA-treated rats before and after removal of the kidneys.

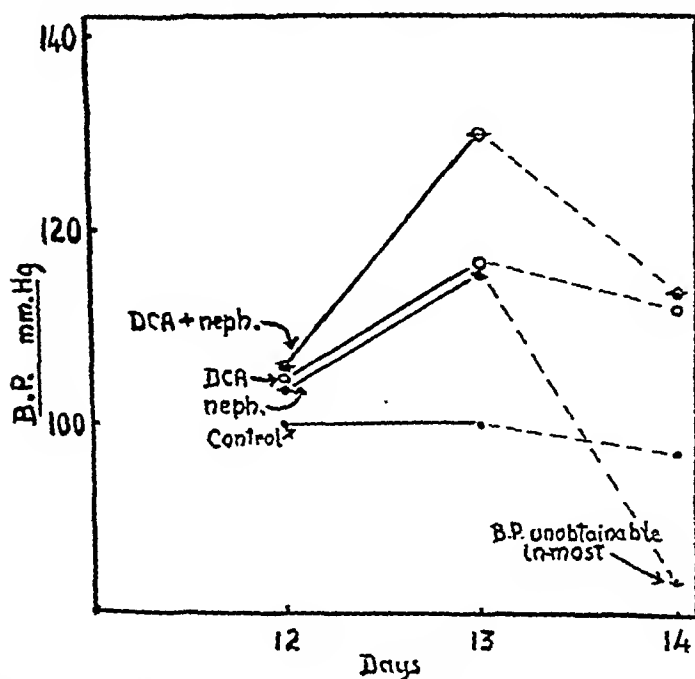


FIG. 4. The blood pressure in DCA-treated rats before and after removal of the kidneys.

Prior to nephrectomy no elevation in blood pressure was observed in any group although 2 days later the DCA-treated animals were beginning to show a rise. Twenty-four hours after removal of both kidneys blood pressure was elevated in both DCA-treated and untreated nephrectomized animals. In the case of the untreated group, this finding was significant but resulted from an elevation of pressure in only 3 of the 9 animals. On the other hand, all 9 DCA-treated nephrectomized animals participated in the observed elevation in this group, an elevation statistically significant not only in reference to the untreated control level but also in reference to the moderate elevation in the two other groups. The 48 hour findings are based on too few data to permit discussion.

DISCUSSION

Earlier workers had observed that when DCA was administered to suitably sensitized animals, hypertension and renal damage occurred. They assumed that the kidneys were primarily involved in DCA hypertension, although such a conclusion was not necessarily warranted by the data. Reinvestigating the problem from a functional approach we drew attention to the absence of renal functional change at a time when hypertension following DCA administration was well established, and pointed to the similarity of this observation to the findings in essential hypertension. Further exploration aimed at both the progression of DCA hypertension and its regression upon cessation of treatment showed, however, that the kidneys are involved from the start in the process. Attention is drawn particularly to those data which show clearly how compensatory hypertrophy may completely mask the presence of interference with renal function.

After removal of both kidneys, an aggravation of the hypertension was observed in DCA-treated animals. It is thus unlikely that the kidney enlargement reflects a stimulated production of renal pressor material, but it seems reasonable to suggest that the kidney is actively concerned with the excretion and possible inactivation of the steroid.

It is also possible to explain these findings according to Grollman's view that the kidney normally liberates an antihypertensive factor (10). In the present state of information, however, it is not possible to distinguish between the idea of a renal antihypertensive factor on the one hand and the renal destruction of a pressor agent on the other.

SUMMARY

Desoxycorticosterone acetate in pellet form was administered for 51 days to albino rats of the Sherman strain which also received 1 per cent saline as drinking water. Treatment was stopped in representative groups at 25, 37, and 51 days so that the regression of blood pressure and renal changes could be ob-

served. It was noted that both the elevation in blood pressure during treatment and its reversal when treatment was stopped were closely correlated with corresponding changes in renal mass. In the time for which the process was studied it did not become irreversible.

Removal of both kidneys from DCA-treated animals aggravated the hypertension, suggesting that the kidneys are actively concerned with the excretion and possible inactivation of the steroid.

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EFFECTS OF PROTEINURIA ON THE KIDNEY*

PROTEINURIA, RENAL ENLARGEMENT, AND RENAL INJURY CONSEQUENT ON PROTRACTED PARENTERAL ADMINISTRATION OF PROTEIN SOLUTIONS IN RATS

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PLATES 32 TO 34

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Renal damage due to cast formation and tubular plugging by protein material, is well known in diseases associated with hemoglobinuria (1), Bence-Jones proteinuria (2), and "albuminuria" (3). The magnitude of this type of damage in Bright's disease is greatly influenced by the globulin content of the urine (4). Whether proteinuria results in renal damage other than that caused by the blockage of tubules, has not been fully determined. Cast formation may sometimes be a result of damage, as well as a cause of damage (5), and tubular lesions which did not appear to be due to obstruction have been observed with hemoglobinuria (6). However, as emphasized in Lucké's review (7), hemoglobinuria as observed clinically is often associated with shock, dehydration, and other factors, which may themselves contribute to the production of renal injury, and in diseases characterized by "albuminuria" or Bence-Jones proteinuria, one cannot always distinguish between possible effects of protein on the kidney, and other manifestations of the disease in question. The use of hemoglobin in investigations designed to determine the effects of protein itself on the kidney, is complicated by the presence of the prosthetic group.

With the advent of the treatment of nephrosis by the parenteral administration of large amounts of protein, usually with an exaggeration of the degree of proteinuria during the period of therapy, the question of the possible harmful effects of proteinuria on the kidney assumed greater importance. The same question also enters into the choice of diet in renal diseases, since the urinary protein often is increased by raising the dietary protein level.

In order to study the effects of proteinuria on the kidney, and particularly to determine whether the passage of protein through the kidney might result in renal damage other than that attributable to tubular obstruction, it was decided to attempt the production in rats of continuous proteinuria of abnormal degree, for extended as well as brief periods of time, and to follow the changes which occurred in renal morphology and composition during and following cessation of treatment, together with the changes in blood, urine, and other organs.

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Preliminary experiments indicated that fairly continuous additions of various proteins to the blood stream, and increases in the amount of protein in the urine could be induced by twice daily intraperitoneal injections of solutions of the proteins. Efforts were made to adjust fluid and electrolyte intake, and the amount of protein administered, in such a manner that tubular obstruction would not occur in the majority of animals, and that the results would not be influenced by differences in urine volume, dehydration, electrolyte imbalance, or shock.

In addition to observing the effects of the protein injections on the kidney, some information was obtained concerning the degree and duration of retention of the various proteins in the plasma, and it was possible to make observations of a preliminary nature bearing on the questions of the mechanism of proteinuria, the passage of protein through the glomerular membranes, and the reabsorption of protein by the tubules.

EXPERIMENTAL

Materials and Methods

Animals.—Two hundred and eighty-five male albino rats of a Sherman strain were given injections as described below. The animals were approximately 24 days old when the injections were started. The maximum variation in initial weight of animals used in any series was less than 10 gm. Approximately 100 large adult rats were used as donors for rat serum which was administered to some of the experimental rats, and about 50 additional rats were employed in auxiliary experiments.

Protein Solutions.—Three per cent solutions of the following proteins, each dissolved in 0.45 per cent sodium chloride solution, were employed for injections in some of the treated groups of animals: gelatin,¹ human albumin,² and bovine gamma globulin.³ Phenyl mercuric borate 0.002 per cent was present in the gelatin solution and was added to the other solutions. In addition to the experiments employing 3 per cent protein solutions, a few rats received a solution containing approximately 6 per cent gelatin in a physiological solution of sodium chloride, with 0.004 per cent phenyl mercuric borate.¹ The solutions were prepared and handled aseptically, and refrigerated when not in use.

Rat Serum.—Rat serum diluted with an equal volume of distilled water (giving approximately a 3 per cent protein solution in half-physiological salt solution), and with 0.002 per cent phenyl mercuric borate added, was used for injections in two groups of animals. The serum administered to the first group was obtained from adult rats decapitated after ether anesthesia of sufficient depth to cause respiratory depression or paralysis. When it appeared that certain undesirable effects of the serum might be due to ether retained by the serum, the serum used for

¹ Purified gelatin in isotonic solution of sodium chloride, oncotic pressure 70 mm. Hg, supplied by the Upjohn Company, Kalamazoo, Michigan, diluted with an equal volume of water. The stock solution containing approximately 6 per cent gelatin was administered without dilution to a few animals of series 9.

² Normal serum albumin (human) concentrated, salt-poor, E. R. Squibb and Sons, supplied by the American Red Cross.

³ Fraction II from bovine plasma, supplied by The Armour Laboratories, Armour and Company, Chicago.

TABLE I
Summary of Experiments

	Series								
	1	2	3	4	5	6	7	8	9
No. of animals receiving following solutions*									
Saline.....	8	6	6	21		18	8	4	6
Sucrose.....	12								
Urea.....			6				8	4	
Casein hydrolysate.....			6	22	3		8		
Gelatin.....	12		6	20	3	18			12†
Human albumin.....		8	6		3	12			
Bovine globulin.....		8	6		3	12			
Rat serum.....							6	4	
Total animals receiving injections (285)....	32	22	36	63	12	60	30	12	18
Length of injection period, days.....	42	35	14	2	7	9	9	4	6
Time of autopsy of animals									
During course of injection period (animals).....	12	6			4			4	
6 to 18 hrs. after end of injection period....	6	4	30	18	8	16	30	8	6
2 wks. " " " " ".....						28			
2 mos. " " " " ".....	12	8							
At intervals of a few days after end of injection period.....				45		16			12
Urine collections for protein determinations									
Near end of injection period.....	x§	x			x	x	x	x	
1 and 2 days after end of injection period..						x			
2 mos. after end of injection period.....	x	x				x			
Determinations of specific proteins in urine .			x			x			
Animals and kidneys weighed at autopsy.....	x	x	x	x	x	x	x	x	
Determinations on blood at autopsy									
Urea.....	x	x	x			x		x	
Total plasma protein (CuSO ₄ method).....	x	x	x			x	x		
Plasma albumin and total nitrogen (Kjeldahl) 						x	x		
Hemoglobin (CuSO ₄ method).....	x	x	x			x	x		
Determinations on renal cortical tissue									
Moisture content.....			x			x	x		
Total nitrogen (Kjeldahl) 			x			x	x		
Tests for specific proteins 		x	x			x			x
Kidneys examined microscopically.....	x	x		x	x		x	x	x

* Solutions previously described in text.

† Six of these animals received the more concentrated gelatin solution (see footnote 1).

§ x indicates that the examination indicated was done.

|| These determinations were done in a limited number of cases.

the second group was obtained after decapitation, from large rats which had been placed in a nitrogen chamber until respiration stopped. These animals frequently remained in an atmosphere with little oxygen for 5 minutes or longer, and convulsions during this period were not uncommon. In both cases, the blood from the donor animals was collected with minimal contamination into tubes by means of paraffin-covered funnels and allowed to clot. The serum was separated promptly by centrifugation, placed in tubes, and immediately frozen. Portions of serum sufficient to last 24 to 36 hours were thawed and diluted as needed for injections, with constant refrigeration of the diluted serum.

Control Solutions.—One per cent urea, and 3 per cent casein hydrolysate,⁴ each dissolved in 0.45 per cent sodium chloride solution, and the 0.45 per cent sodium chloride solution alone, were administered individually to control groups of animals. Phenyl mercuric borate 0.002 per cent was added as a preservative. The solutions were aseptically prepared and handled, and refrigerated between injections. In some of the later experiments, distilled water rather than half-physiological salt solution was used in the preparation of the urea and protein hydrolysate solutions in order to decrease peritoneal irritation.

General Plan of Experiments.—Comparable groups of the young rats were given, for various periods of time, twice daily intraperitoneal injections, 1 cc. per 10 gm. of body weight at each injection, of the protein, serum, and control solutions. The different series of injections, and the determinations which were done on the animals of each series are summarized in Table I. The injections were made without anesthesia, through a 21 gauge needle, care being taken to maintain aseptic conditions and to avoid injury of the liver and intestines. In order to insure an equal fluid intake and the elimination of urine of comparable volume and concentration by the animals of the different groups, *no fluid* except that administered in the injections already described was allowed. A stock diet⁵ was offered *ad libitum*. For several days prior to and during the urine collections, a low residue synthetic diet⁶ was substituted for the stock diet in order to decrease the bulk of the feces. Because vitamin-free injected materials were to some extent utilized in place of food by animals of some of the groups, complete vitamin supplements⁷ were offered *ad libitum* to all animals. A complete salt mixture⁸ was also supplied separately so that the animals could adjust their own salt intake to some extent. The animals were weighed at intervals of a few days and the amounts of injected materials were increased as indicated. Urine was collected from time to time for protein determinations. Animals from various groups were autopsied at intervals during, at the end of, and at periods following terminations of injection periods. Blood was collected for study, weight of kidneys was determined, and kidney tissue was preserved for chemical and histological study. Animals not autopsied during, or at the end of, injection periods were given water *ad libitum* beginning 24 hours after the final injection. The occasional animals which became seriously ill or died were not included in the determinations of kidney size.

Urine Collections.—Urine was collected by means of metabolic cages placed above large paraffin-coated funnels which drained into flasks containing a little toluene. Because of the

⁴ Amino acids—I. C., lyophilized (acid hydrolysate of casein with added tryptophane) supplied by Biochemical Division, Interchemical Corporation, Union, New Jersey.

⁵ Purina fox chow.

⁶ The diet employed was the same as that described elsewhere (8), with 0.3 per cent choline chloride added. The changes in the diet did not appear to influence appreciably the kidney weight-body weight ratios or the amount of protein in the urine. The dietary treatment of protein-treated and control groups was the same in each experiment.

⁷ The vitamin mixture was the same as that used in the synthetic diet (8), with added choline chloride.

⁸ Osborne-Mendel salt mixture No. 2, Eimer and Amend, New York.

small size of the animals, from 3 to 6 rats were placed together in each cage. Collections were made for periods of from 12 to 24 hours. When injections were continued during the periods of urine collections the animals were not returned to the metabolic cages until leakage from peritoneal cavity, if present, had ceased. Since fairly uniform results were obtained, it was thought that subsequent leakage did not occur. Because of technical difficulties, the collections were only approximately quantitative. As indicated in Table I, urine collections were made at various times after termination of injections, as well as near the end and sometimes at earlier stages of the injections.

Urine Examinations.—The urine specimens were cleared by centrifugation, and filtered if necessary. Volume was recorded, and protein concentration determined by the Shevky-Stafford (9) or the biuret (10) method. Gelatin was precipitated by addition of tungstate or Tsuchiya's reagent after removal and estimation of other proteins by addition of an equal volume of 10 per cent trichloroacetic acid. Human albumin was estimated in a few cases by determination of the turbidity produced by addition of antiserum prepared from rabbits.⁹ Bovine globulin was tested for qualitatively by serological precipitation, and the relative globulin content of urines from various groups was estimated by comparing the precipitates formed by adding equal volumes of 44 per cent sodium sulfate, after adjusting pH of the urine specimens to 7.4 (11).

Urine sediment was examined microscopically in a few instances.

Blood Examinations.—At the time of autopsy, during, or sometime after termination of the injections, blood was collected into heparin-containing flasks after decapitation of the animal. Total plasma protein and hemoglobin levels were estimated from specific gravity determinations by the copper sulfate method (12). Urea was determined by the hypobromite method (13). Total plasma nitrogen and plasma albumin determinations were done in a few instances by a micro Kjeldahl method (14), using pooled plasma from the various groups.

Examination of Organs.—After comparable animals of different groups had been weighed, killed by decapitation, and thoroughly drained of blood, the peritoneal cavities were inspected for free fluid, and for evidence of peritoneal irritation, infection, or injury to organs. When fluid was present in the peritoneal cavity, the weight of the animal was corrected by deducting the weight of the free fluid. Tissues were observed for evidence of dehydration or overhydration. The size, color, and other gross features of kidneys, liver, and spleen were specifically noted. Differences in size and shade of kidneys were most impressive when the organs from comparable control and protein-injected animals were compared directly by placing the kidneys side by side. The kidneys and sometimes the liver and spleen were removed for weighing, and tissue was preserved for histological study of gross and microscopic sections, and for total nitrogen and moisture determinations.

Determination of Relative Weight of Kidneys.—The animals of the different groups were of approximately the same weight at the beginning of the injection periods, and the growth rate of all the animals was fairly uniform, at least for several weeks. In experiments which were continued for a number of weeks, there was sometimes considerable variation in weight of the animals at the time of autopsy. In the early experiments, the kidneys were removed without attached tissue, but with capsules in place, and were weighed accurately. After it was observed that some of the injected solutions produced significant thickening and increase in weight of the renal capsules, the capsules of all kidneys were removed carefully before weighing. In order that the kidneys of various animals might be compared more accurately, the ratio of kidney weight to corrected body weight was determined in each case. In each series of experiments, animals of various groups were treated similarly. In most cases, autopsies were

⁹ We are indebted to Dr. Henry G. Kunkel for the determinations of human albumin in urine and renal cortical tissue.

performed about 18 hours after the last injection, with no oral fluid during the intervening period. In some of the early experiments, however, the time interval between the last injection and autopsy was less than 18 hours.

Histological Examinations of Kidneys and Other Organs.—After the kidneys were weighed, they were sectioned along the median sagittal plane, and direct comparisons made of kidneys of animals of various groups. Kidney tissue was then fixed in alcohol-formol-acetic acid,¹⁰ or sometimes in 10 per cent neutral formalin or in absolute alcohol. Paraffin sections were prepared as routine, and stained for microscopic examination with hematoxylin and eosin or with Giemsa's stain, and frequently with Mallory's connective tissue stain. A few sections from kidneys of gelatin-treated animals were fixed in absolute alcohol and stained with eosin and with several other stains in absolute alcohol, without passage through aqueous solutions. Frozen sections of formalin-fixed tissue were stained for fat in a few instances. Liver sections from some animals and sections of other organs from occasional animals also were studied microscopically.

Determinations of Water and Other Constituents of Cortical Tissue.—Cortical tissue was prepared by bisecting decapsulated kidneys and removing the medullary portion. The cortical tissue was then pressed against blotting paper to remove free fluid present in tubules and blood vessels.

Moisture determinations were made on kidneys from most of the animals. This was done by placing the cortical tissue from one-fourth to one-half of a kidney in a weighing bottle, accurately determining the wet weight of the tissue, drying in an oven at 104°C. for 6 hours, and again weighing. The percentage loss of weight by the wet tissue on drying was then calculated.

After tissue had been taken for sections and for moisture determinations, the remaining cortical tissue was placed in tubes, weighed, and frozen. Total nitrogen determinations were performed on this tissue in a few cases by a Kjeldahl method (14). In some other cases, the tissue was ground in water or saline with sand or by means of a Potter homogenizer. The solutions were cleared by high speed centrifugation, and gelatin, human albumin, and bovine globulin were precipitated as already described for the proteins in urine. Results on kidney tissue from control and protein-treated animals were compared in each instance. In a few cases, the protein injections were discontinued and the animals given water for 2 to 7 days before autopsy in order to eliminate most of the injected protein from blood and urine, small amounts of which were retained by the renal tissue. Although a few quantitative estimations of the specific protein were made, the results were of only qualitative significance because certain factors were not well controlled.

RESULTS

Most of the animals which received injections of the various substances remained well and grew fairly normally. The animals which received injections for the longer periods grew from weanlings to rats weighing approximately 200 gm. without receiving any fluids by mouth. Disturbances in health and growth of animals due to intraperitoneal infections or injuries produced at the time of injections, were rarely encountered. There was no evidence of sensitization of the protein-injected animals to the proteins, though temperatures of the injected animals were not determined following injections and sera were not examined for antibodies against the proteins. The impression was obtained that the animals which were given gelatin were less excitable and struggled less

¹⁰ Eighty per cent alcohol 900 cc., 40 per cent formalin 50 cc., and glacial acetic acid 50 cc.

during injections than those of other groups. In the early experiments, the animals receiving protein hydrolysate appeared to be less able than animals of other groups to tolerate periods of more than 12 hours without an injection of fluid. This difference was partially removed by administering the protein hydrolysate in water rather than in sodium chloride solution, and by offering a complete salt mixture separately.

Data obtained from groups of animals injected with rat serum are included in tables and charts along with data from other groups. However, since the experiments with the homologous serum were not considered entirely satisfactory, they will be discussed separately.

Absorption of Injected Materials from Peritoneal Cavity.—The control (non-protein) solutions were absorbed somewhat more rapidly from the peritoneal cavity than were the protein solutions. In most experiments, even the protein solutions were fairly completely absorbed during the period from one injection to the next, and only in the case of one group receiving the usual 3 per cent solution of gelatin (series 4), the group receiving 6 per cent gelatin (series 9), and particularly the groups receiving rat serum did intraperitoneal accumulations of injected material present a serious problem. The animals receiving urea became dehydrated and the peritoneal surfaces did not have the normal moist appearance, though the hemoglobin levels did not appear to rise appreciably.

Changes in Plasma Proteins and Hemoglobin Levels Resulting from Injections.—Plasma protein and hemoglobin levels, which were determined in animals of the various groups at the time of autopsy of the animals, during the course of, and at intervals following the termination of injection periods, varied somewhat from group to group, and with the length of the injection period, and with the period of time elapsing between the last injection and the autopsy of the animal, but, qualitatively, the results were similar. Data obtained from animals of series 3, 6, and 7, are presented respectively in Tables II, III, and IV. Little or no change in serum protein or hemoglobin levels was observed with gelatin injections. Unfortunately studies were not done on animals of series 4 receiving the usual gelatin solution, or on those of series 9 receiving the more concentrated gelatin solution, some of which retained fluid in the peritoneal cavity, and some of which developed severe tubular damage. Albumin produced significant elevations in total plasma protein, plasma albumin, and in the ratio of albumin to globulin, with a coincident decrease in the hemoglobin level. Globulin induced an even greater rise in total plasma protein and plasma globulin, but the decrease in hemoglobin concentration was of about the same magnitude as with albumin. After termination of injections, the plasma protein and hemoglobin values returned fairly promptly toward normal.

Effect of Injections on Blood Urea Levels.—The animals receiving injections of control and protein solutions, including those of the long term experiments were found at the time of autopsy to have blood urea nitrogen values ranging between 12 and 28 mg. per cent, with the majority in the lower two-thirds of the

range, and there was no consistent difference between control and protein-treated animals. Some of the higher values were found in animals which appeared ill, but neither illness nor high urea nitrogen values were more common in protein-treated than in control animals, and neither could be attributed with any degree of certainty to specific effects of the injected materials on the kidneys. In animals given continued injections for prolonged periods and then observed for several weeks or months on a normal regime of food and water by mouth, the blood urea nitrogen values were still found to be normal. Blood urea levels were not determined in series 4 and 9 where retention of fluid in the peritoneal cavity, and renal damage, were observed in some of the animals receiving gelatin.

TABLE II
Total Plasma Protein and Hemoglobin Levels of Rats of Series 3

Injected solutions*	Total plasma protein	Hemoglobin
	<i>gm. per cent</i>	<i>gm. per cent</i>
Saline	6.5	10.6
Urea	6.2	9.9
Casein hydrolysate	6.5	11.0
Gelatin	6.4	10.1
Albumin	8.5	8.4
Globulin	9.5	8.3

The animals were autopsied about 6 hours after the last injection, following an injection period of 2 weeks. Each figure represents an average from specific gravity determinations on 3 rats by the CuSO_4 method.

* Solutions previously described in text.

Influence of Injections on Proteinuria and Urine Output.—Rapidly occurring diuresis after each injection was noted in animals receiving injections of urea, and the volume of urine excreted in 24 hour periods by these animals was usually somewhat greater than that excreted by comparable animals of other groups, despite the fact that the fluid intakes of comparable animals of all groups were equal. The rate of urine excretion was more uniform throughout the 24 hour periods in the protein-treated animals than in those receiving saline and amino acids, but the total urine volumes of animals of all these groups were approximately equal.

Urine from saline-injected animals always contained small amounts of protein, nearly always less than 1 gm. per liter, and often in the range of 0.5 gm. per liter or less. The total protein excreted per day varied with the size and age of the rat, but after an injection period of 2 weeks, starting with weanling rats, the amount was usually found to be between 1 and 2 mg. per rat per 24 hours. The amounts of protein excreted by the animals receiving amino acids

TABLE III
Plasma Protein and Hemoglobin Levels of Rats of Series 6

Injected solutions*	18 hrs. after final injection			2 days after final injection		5 days after final injection		10 days after final injection		
	Plasma protein†	(Plasma protein) A/G‡	Hb‡	Plasma protein†	Hb‡	Plasma protein†	Hb‡	Plasma protein†	(Plasma protein) A/G‡	Hb‡
	gm. per cent		gm. per cent	gm. per cent	gm. per cent	gm. per cent	gm. per cent	gm. per cent		gm. per cent
Saline	5.0	(6.0)	12.2	5.5	12.7	5.7	12.6	5.7	(5.7)	11.4
Casein hydrolysate	5.2	—	13.3	—	—	—	—	—	—	—
Gelatin	5.3	(4.8)	12.1	—	—	5.7	12.7	5.4	(5.5)	10.8
Albumin	6.5	(6.7)	10.2	5.8	11.7	—	—	5.4	(5.3)	10.9
Globulin	8.6	(9.5)	10.5	8.0	13.0	6.5	14.0	6.0	(5.9)	11.2
		0.33							1.3	

After an injection period of 9 days, the animals were autopsied at intervals as indicated in the table. Animals were given water *ad libitum* beginning 24 hours after the final injection.

* Solutions previously described in text.

† Average values calculated from specific gravity determinations on a total of 40 rats by the CuSO₄ method.

‡ Single determinations on pooled plasma, using Howe separation and micro Kjeldahl.

TABLE IV
Total Plasma Protein and Hemoglobin Levels in Rats of Series 7

Injected solution*	Total plasma protein	Hemoglobin
	gm. per cent	gm. per cent
Saline	5.7	12.5
Urea	5.6	12.8
Casein hydrolysate	6.0	11.9
Rat serum	7.8†	11.4

Animals were autopsied about 18 hours after the last injection, following an injection period of 9 days. Each figure represents an average value calculated from specific gravity determinations on 6 rats by the CuSO₄ method.

* Solutions previously described in text.

† A single determination on pooled plasma indicated that the albumin fraction was only slightly elevated.

and urea were in the same range as that of the saline animals, though the values for the urea-injected animals appeared to be consistently slightly higher than those of the other control groups, perhaps due to the diuretic effect. Additions

of sodium sulfate according to the method for globulin precipitation, to urines from the control groups, produced not more than a faint cloud, and most of the protein was presumably albumin. Urine specimens collected 1 to 2 weeks after starting injections, from the protein-treated animals, almost always contained more than 1 gm. of protein per liter of urine. The animals receiving albumin sometimes excreted as much as 5 gm. per liter or more over the 24 hour period. Globulin induced less marked elevations in urinary protein levels, while gelatin injections were usually accompanied by greater degrees of proteinuria than those observed with albumin.

The increase in urinary protein in animals injected with gelatin was due principally to the presence in the urine of gelatin; the other urinary proteins (precipitated by an equal volume of 10 per cent trichloroacetic acid) did not appear to be regularly or markedly altered. Gelatin appeared in the urine and was occasionally seen in the glomerular capsules before there were appreciable changes in kidney size or visible alterations in the tubular cells. In animals injected with human albumin, preliminary determinations of the human albumin in the urine by the serological precipitin reaction suggested that only a part, probably less than one-half of the urinary protein increment was composed of the injected human albumin. The remainder was presumably rat protein, probably chiefly albumin. As in the case of the albumin, it appeared unlikely that the injected globulin was present in the urine in sufficient quantities to account for all the increase in urinary protein which accompanied injections of that protein.

In animals receiving injections of albumin and globulin, the urinary protein levels, like the plasma protein levels already discussed, remained definitely elevated for at least several days after termination of the injections. However, when urine specimens from the various groups receiving proteins were examined after periods of 2 weeks to 2 months, the urinary protein levels were found to be within the same range as in the specimens from comparable control animals.

Examination of urinary sediment from protein-treated animals revealed no abnormalities.

Gross Changes in Organs Resulting from Injections.—Examinations of the peritoneal cavities sometimes revealed adhesions between liver or intestines and omentum or adjacent organs, due to puncture wounds produced at the time of injections. Significant peritoneal infections were never recognized, if present. Prolonged injections of urea solution, and particularly of protein hydrolysate solution, appeared to produce peritoneal irritation, with thickening and opacity of peritoneal surfaces including hepatic and renal capsules. In the case of urea, the peritoneal surfaces were often less moist than with the other solutions. Saline and the 3 per cent protein solutions produced no peritoneal changes. However, the more concentrated gelatin solution used in series 9 produced definite peritoneal thickening, with rounding of the liver edges.

Occasional animals, without respect to treatment, were observed to have large dark spleens.

It was noted during the early periods of the study that the kidneys of animals which had received repeated injections of gelatin appeared enlarged and pale. The correctness of this observation was confirmed by side by side comparisons of the kidneys of control and gelatin-treated animals (Figs. 1 and 3). When sagittal sections of kidneys from gelatin-injected animals were compared with similar sections from comparable control kidneys, it was further evident that the enlargement of the kidneys from animals receiving gelatin was principally in the cortical portion. The cortical tissue appeared pale particularly through the outer two-thirds of its depth, and was separated from the pale medullary tissue by a narrow dark band at the corticomedullary junction.

In experiments employing albumin and globulin, similar but usually somewhat less marked renal enlargement was noted (Figs. 2 and 3). Paleness of some degree was seen frequently with injections of these proteins, particularly when enlargement of considerable degree occurred, but it was usually much less than with gelatin.

No gross alterations, other than the differences in size and depth of color, were noted in any of the kidneys, and the pelves and ureters always appeared normal.

There appeared to be no changes in the liver or spleen attributable to injections of protein, though these organs were accurately weighed in only a few cases. No changes were noted in the adrenal glands, which were not studied extensively.

Change in Relative Size of Kidneys Due to Injected Substances.—Since there was always some variation in size of the experimental animals at the time of autopsy, the feasibility of expressing the relative kidney size as the ratio of the weight of both kidneys divided by the weight of the animal, was investigated. It was found that this ratio was quite constant over the range of variation in animal size encountered in the individual experiments, particularly in those of not more than a few weeks' duration. This ratio, multiplied by 100, decreased steadily with growth of the animals, from approximately 1.25 in the youngest rats autopsied to about 0.67 in the rats of series 1 which were autopsied after being observed for 2 months at the end of the 6 weeks injection period.

In the early experiments, the kidneys were weighed without decapsulation. However, after the introduction of urea, amino acid, and rat serum solutions, it was observed that these substances caused sufficient thickening and increase in weight of the renal capsules to alter appreciably the kidney weight-body weight ratios, and in subsequent studies the kidneys were carefully decapsulated before weighing.

The animals receiving urea and protein hydrolysate solutions, like those receiving saline alone, exhibited little change in relative kidney size. The kidney weight-body weight ratios of the animals receiving urea were usually slightly

elevated compared with those of the other control groups, perhaps because of a decrease in body weight due to dehydration.

Protein solutions uniformly caused an increase in relative kidney size and weight. The most rapid enlargement appeared to occur in the early part of

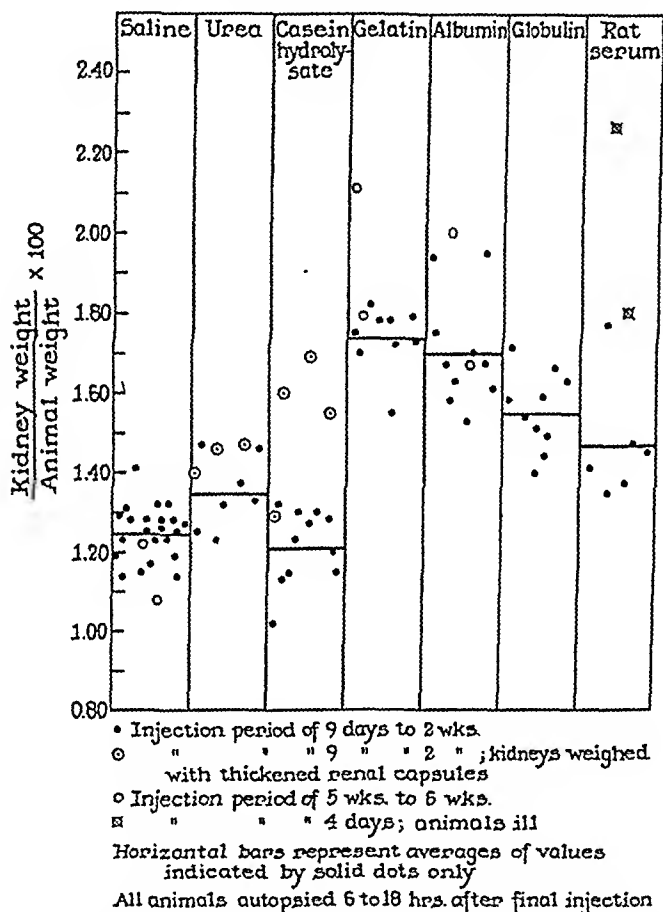


CHART 1. Relative renal size of comparable rats injected with various protein and control solutions.

the injection periods, but the enlargement was maintained as long as the injections were continued.

Gelatin caused the most rapid and usually the most marked increase in kidney size. Definite enlargement occurred within a few days, and perhaps within 1 day. Albumin ultimately caused almost as great enlargement as gelatin but the enlargement appeared to develop more slowly. Globulin produced less enlargement than gelatin or albumin.

Chart 1 shows the results of determinations of relative renal size which were made in the animals receiving injections for 9 days or longer and autopsied within 1 day after the final injection. The animals were from series 1, 2, 3, 6, and 7, with the two ill animals from series 8. Chart 2 contains further data from series 4 on the enlargement produced by short term injections of gelatin.

Reversibility of Renal Enlargement after Termination of Injections.—Chart 2 shows the changes in relative kidney size over a period of 8 days in rats of series 4, given 3 single injections of gelatin solution, compared with those of rats given injections of saline and casein hydrolysate. For some unknown reason,

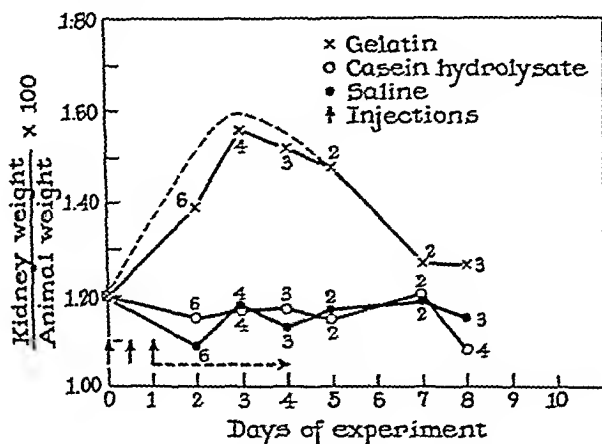


CHART 2. Showing changes in relative renal size on successive days produced by three injections of gelatin, compared with control injections. Absorption of gelatin was unusually slow, as indicated by horizontal arrow, and weight of animals was not corrected for weight of peritoneal fluid. Broken line indicates approximate correction. Numerals indicate number of observations. Average weight of gelatin-treated animals autopsied on various days was: 2nd day, 46 gm., 3rd day, 48 gm., 7th and 8th days, 58 gm. Data from series 4.

possibly because the animals were autopsied at an earlier age than most, it was found that the gelatin solution had not been as completely absorbed as usual, and fluid was present in the peritoneal cavities of many of the rats for 2 or 3 days after the final injection. This continued absorption probably accounted for the further increase in relative kidney size after injections were discontinued. The chart demonstrates that a prompt increase in the relative size of the kidneys of the gelatin-treated animals occurred, followed by a prompt return toward normal as soon as the injections were discontinued and the gelatin was all absorbed from the peritoneal cavity. Evidence of some degree of renal damage was observed in the kidneys of several rats of this group, as will be discussed in more detail later.

Chart 3 shows the changes in the kidney weight-body weight ratios, during

the 10 day period following termination of a 9 day injection period, in animals receiving various protein solutions. The final injection is indicated by vertical arrow. Protein solutions had been almost completely absorbed when the first animals were autopsied on the 10th day.

The decrease in kidney weight-animal weight ratio after termination of protein injections, particularly when observations were made over a considerable period, was due in part to a dilution of the kidney weight increment which resulted from the injections, due to normal growth of the kidneys. In addition to this effect, however, there was a prompt decrease in the absolute difference

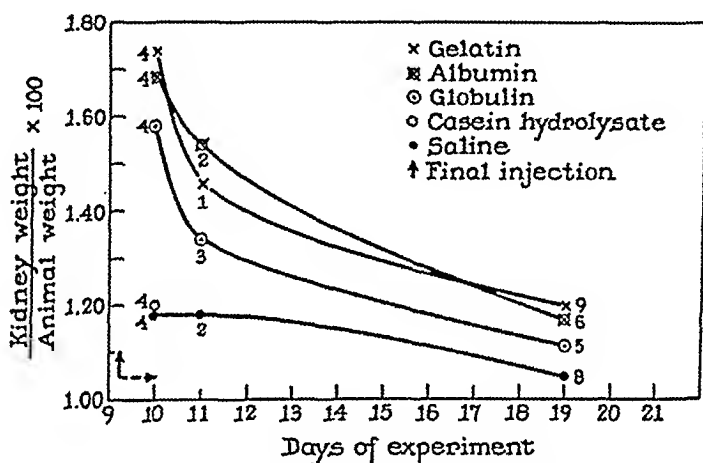


CHART 3. Showing return of kidneys of protein-treated animals toward normal size, following termination of a 9 day injection period. Data are from series 6. Numerals on chart indicate number of observations at each point. Horizontal arrow indicates duration of presence of protein solutions in peritoneal cavities of animals. Average weights of various groups of animals autopsied at different points on chart were: 10th day, 51 to 52 gm., 11th day, 52 to 56 gm., 19th day, 75 to 86 gm.

in size between the kidneys of protein-treated and control animals. Charts 2 and 3 indicate that some residual enlargements of the kidneys of the protein-treated groups probably remained at the end of the periods of observation. In series 1, there appeared to be slight enlargement of the kidneys of the gelatin-treated animals 2 months after the injections were discontinued. However, since any residual enlargement becomes a progressively smaller portion of the total renal tissue as the animals grow older, and greater variation in animal size occurs, the long term observations on small groups of animals were of doubtful significance. It can only be concluded that there was a prompt return of the kidneys of the protein-injected animals in the direction of normal size, after protein injections were discontinued.

Composition of Kidneys of Animals Receiving Various Solutions.—After it was apparent that injections of protein solutions resulted in renal enlargement,

it seemed of importance to determine whether the enlargement was due simply to an increase in the water content of the renal tissue, or whether it was due to an increase in material of approximately the same solid content as normal renal tissue. Water determinations were used to answer this question. Since the enlargement appeared to involve chiefly the cortical portion of the kidney, cortical tissue was used for the determinations. It is evident from data in Table V, representing water determinations on kidney tissue from a total of 70 animals, that only a slight part, if any, of the enlargement was due to an increase in the proportion of water to solids in the renal tissue of the protein-treated animals. The increase in weight was due to addition of water and solids in almost the same proportion present in normal kidney tissue. There was a slight increase in average relative water content of the tissue from animals

TABLE V
Moisture Content of Renal Cortical Tissue of Rats from Series 3, 6, and 7

Injected solutions*	Moisture	
	18 hrs. after final injection	10 days after final injection
	<i>per cent</i>	<i>per cent</i>
Saline	77.2	77.3
Urea	76.8	—
Casein hydrolysate	76.8	—
Gelatin	78.0	77.2
Albumin	78.8	78.3
Globulin	76.9	77.5
Serum	77.5	—

Injections were continued for 9 days to 2 weeks.

* Solutions previously described in text.

receiving albumin, and possibly in that of those receiving gelatin, 18 hours after termination of the injections. The 10 day values are less reliable because of the smaller number of determinations and greater spread of individual values.

Total nitrogen determinations on a few kidneys of various groups gave approximately similar results in most of the control and protein-treated animals and indicated that the protein concentration was probably normal in the protein-treated animals. The increase in kidney weight was apparently due in most instances to addition of water and protein in nearly the same ratio existing in normal kidneys.

Injected Protein in Renal Tissue.—Homogenates of renal cortical tissue from gelatin-injected animals were usually found to contain definitely more material resembling gelatin, in that it was precipitated by tungstate but not by a final concentration of 5 per cent trichloroacetic acid, than was found in the renal tissue from other groups. This was true even when the determinations were made several days after gelatin injections were discontinued. However, efforts

were not made to insure quantitative extraction of gelatin or other specific proteins from the renal tissue and quantitative determinations of gelatin were not made in most cases. The desirability of further studies was evident when, during the course of the experiments, it was found that kidney tissue from *control* animals sometimes contained more than the minimal amounts of "gelatin" observed in the early experiments.

In extracts of kidneys from rats that received human albumin and bovine globulin, serological precipitin tests showed the presence of these injected proteins. The amounts of extracted human albumin were much smaller than necessary to account for the total enlargement of the kidneys associated with injections of that protein, and it was not established beyond question that the results could not be accounted for on the basis of human albumin in the blood and urine retained by the renal tissue. Quantitative determinations of the globulin were not attempted.

These results were regarded as suggestive but not conclusive evidence that the renal parenchymal cells contained quantities of the injected proteins.

Microscopic Observations.—No abnormalities or differences were noted on microscopic examination in the kidneys of animals receiving saline, (Fig. 4), urea, or protein hydrolysate.

Kidneys from animals receiving gelatin showed, in addition to the enlargement and paleness apparent on gross examination, rather marked alterations in the cells of the convoluted tubules, particularly in the outer two-thirds of the cortex (Figs. 5 and 6). The proximal tubules were probably more markedly involved than the distal segments. The cells of the convoluted tubules were enlarged and contained what appeared to be clear spaces in the cytoplasm. These clear spaces were observed in sections of tissue that had not been exposed to aqueous solutions, as well as in those subjected to the routine staining procedures. There was no increase in fat content of the cells as shown by fat stains. The lumina of the convoluted tubules appeared to be decreased in width and in some cases they were almost closed, due to encroachment by enlarged cells.

Although the kidneys of the animals receiving solutions of albumin and globulin were definitely enlarged in the gross, on microscopic examination no definite and consistent changes were noted which might have been responsible for the enlargement. There appeared to be slight cytoplasmic alterations in the cells of the convoluted tubules in some cases. Increases in size of the cells of the convoluted or other tubules, or changes in caliber of the tubular lumina, if present, were not of sufficient magnitude to be recognized, and there was no other apparent explanation for the increase in bulk of the cortical tissue. In most of the animals of the protein-treated groups, no changes suggestive of an increased production of new cells in the cortex, were observed; mitotic figures were noted in the tubules of some animals autopsied early in the course of gelatin injections.

In the kidneys of some animals receiving proteins, protein material was visible in occasional glomerular capsules and tubular lumina. Rare isolated tubules in the region of the straight segments appeared to be plugged with protein material but similar tubules also were seen from time to time, but perhaps less frequently, in control kidneys. No abnormalities were noted in glomeruli, blood vessels, or interstitial tissue, even in animals treated and observed for prolonged periods. No changes suggestive of sensitization of the animals to the injected proteins were observed.

Three or four gelatin-injected animals of series 4—the series in which absorption of the injected 3 per cent gelatin solution was unusually slow—differed further from most of the protein-treated animals in that definite evidence of renal damage was observed on microscopic examination of the kidneys. These animals in which definite damage was recognized were among those which were autopsied from 2 to 5 days after gelatin injections were discontinued. The morphological alterations were much more extensive in the kidneys of one of the animals (Figs. 7 and 8) than in those of the others. These alterations were localized principally in the inner cortical and outer medullary zones where glomerular capsules and tubules appeared dilated and contained some protein material, and the tubules were lined by flattened epithelium containing mitotic figures, large hyperchromatic nuclei, and basophilic cytoplasm. Similar dilation of glomerular capsules and tubules, not accompanied by evidences of tubular necrosis or regeneration, was present at the time of autopsy in the kidneys of some of the gelatin-treated animals of series 9, particularly those receiving the more concentrated solution. In the kidneys of the majority of animals receiving each of the 3 per cent protein solutions, no changes were observed which could be considered evidence of renal damage, unless the cytoplasmic alterations in the tubular cells induced by gelatin are interpreted as evidence of damage.

In the kidneys of animals receiving gelatin, the appearance of the cells of the convoluted tubules gradually returned toward normal after injections were discontinued, with the clear spaces decreasing in size and finally disappearing. While there was considerable individual variation, the morphological alterations largely disappeared in most cases within about a week, during which time the renal enlargement and the "gelatin" in the renal tissue also decreased.

During the periods following the injections no impressive renal cytologic changes were observed in the animals receiving the control solutions, or those receiving albumin or globulin.

No consistent microscopic changes were observed in the livers of any of the groups of protein-treated animals.

Observations on Animals Treated with Rat Serum.—Since renal enlargement and an increase in urinary protein excretion were induced by injections of heterologous proteins, it was desirable to determine whether similar effects would result from injections of homologous proteins. The experiments with

the proteins already discussed, particularly the rather preliminary studies on the specific proteins in the urine, suggested that the increased proteinuria was not dependent on the heterologous nature of the injected proteins but would be produced by homologous proteins as well. Because purified rat proteins were not available, it was decided to investigate the effects of injecting homologous serum. Some of the data from these studies have been included in the tables and charts already presented, in order to facilitate comparisons with data from other groups. However, for several reasons, separate discussion of the experiments with rat serum seemed desirable.

In the first experiment with rat serum (series 7 of Table I), the animals with the possible exception of one, appeared quite well throughout the experimental period. Some enlargement of the kidneys occurred (Chart 1), but this was of less magnitude than with the other protein solutions. At the time of autopsy, more than 12 hours after the final injection, large amounts of unabsorbed fluid were found in the peritoneal cavities of some of the animals. In experiments where solutions of gelatin, albumin, or globulin had been injected for similar periods of time, absorption had nearly always been more complete. In addition, the serum-injected rats showed evidence of peritoneal irritation with definitely thickened renal capsules, not observed in the animals receiving the 3 per cent solutions of single refined proteins. Furthermore, urine collections made on the last 2 days of the experiment, which were the only specimens collected, showed no definite increase in protein content as compared with control specimens.

It was thought that perhaps ether retained in the rat serum (the donor animals were deeply anesthetized with ether) caused progressive peritoneal irritation with a corresponding decrease in the rate of absorption of injected serum, and that this explained the fluid retention in the peritoneal cavities, the low levels of urinary protein excretion, and the absence of greater renal enlargement. A second experiment (series 8) was then performed, using rat serum which was collected from donor animals after they were rendered unconscious by anoxia in a nitrogen chamber. In this experiment, the serum-treated animals retained much of the injected serum solution in the peritoneal cavities from the beginning, excreted little urine, and became definitely ill with elevated blood urea levels. Two of the four animals died on the 4th day after becoming quite pale and cold. The other two animals were autopsied and found to have greatly enlarged kidneys. Urine collected on the 3rd and 4th days from animals of this group contained considerably increased protein levels.

Microscopic Observations on Serum-Treated Animals.—Sections from the kidneys of all but one of the animals of the first serum-treated group appeared entirely normal, with the exception perhaps that the tubular lumina contained more protein material than normal. Changes similar to those observed in the gelatin-treated animals of series 4 with the severer forms of injury, were seen in milder degree in sections of the kidneys of one rat of this first group.

When the greatly enlarged kidneys of the second serum-treated group were sectioned, a discrete band occupying the region about the corticomedullary junction, which obviously contained large amounts of calcium (Fig. 9), could be seen with the unaided eye. Microscopic examination of sections confirmed the presence of extensive tubular damage with necrosis and calcification in the zone including the deepest portion of the cortex and adjacent medullary tissue (Figs. 10 and 11). Many glomerular capsules contained protein precipitates, and the tubules in the area beneath the zone of calcification were dilated, lined by flattened epithelium, and contained large amounts of deeply staining protein material.

DISCUSSION

Only the inconclusive data of Bordley and Richards (15) and Walker *et al.* (16) are available concerning the protein content of the normal glomerular filtrate, though the constant occurrence under uniform conditions of fairly uniform levels of protein in the urine of the normal rat (17) appears to be presumptive evidence that the glomerular filtrate of that animal contains some protein. Accumulated evidence (18-21) indicates that the glomerulus is at least the chief source of the protein which appears in the urine under the various abnormal conditions which have been studied; it does not eliminate the possibility, however, that all or a part of the protein in the urine under certain circumstances may be present as a result of defective tubular reabsorption of protein normally or abnormally filtered from the glomeruli (22). It was evident from examination of the sections in the present study that the increased proteinuria which occurred with the protein injections was associated with an increased amount of protein in the glomerular filtrate. Furthermore, gelatin appeared in the urine prior to the development of the characteristic changes in the tubular cells which were presumably associated with the presence of gelatin in the cells.

Because of the chronic nature of the experiments there may have been a compensatory increase in hemoglobin levels of the protein-injected animals, and the hemoglobin levels at the end of the injection periods may not have adequately reflected the magnitude of the blood volume changes.

Proteinuria has been observed in dogs following injections of homologous plasma (23), and in human patients without renal disease following injections of homologous albumin (24). In the present experiments, an increase in urinary protein excretion was observed in some, but not in all the rats receiving homologous serum; interpretation of the results was complicated by the occurrence in some of the animals of incomplete absorption of fluid, oliguria, and renal damage.

The rather cursory study of the specific proteins in the urine of the rats seemed to indicate that increased amounts of homologous protein, as well as quantities of the heterologous protein, appeared in the urine following injec-

tions of human albumin and bovine globulin, which accumulated in the plasma, but not following gelatin which was passed into the tubular lumina to at least as great extent, but which caused less hemodilution than the other proteins. These observations might be interpreted as suggesting that the filtration of a protein present in the plasma may be increased, with no increase in the concentration of the protein, by changes in dynamics of the glomerular circulation associated with an increase in blood volume, though other explanations of the observations are conceivable.

Reversible renal enlargement, comparable in degree and rapidity of development to that observed in the present experiments following the injections of proteins, has been shown to occur in rats on diets containing high levels of various proteins, including casein (25-27). This renal hypertrophy under conditions of high protein diets has usually been considered a result of an increase in renal work associated with the metabolism and excretion of products of protein digestion. Some degree of renal enlargement due to feeding of urea itself has been observed by some investigators (28), but others have obtained essentially negative results (25). Renal injury also has occurred under certain circumstances with high protein diets (29, 30).

In the present study, renal enlargement such as occurred with the protein injections, was not observed following injections of urea or casein hydrolysate. It perhaps is possible, in spite of careful planning of the experiments, that differences in food intake, nutritional state, or hydration of tissues, might have been responsible for the difference in relative renal size which developed in the animals receiving protein hydrolysate, as contrasted with those receiving protein itself. However, there was no evidence from examination of the animals that such differences were present to an important degree.

Another explanation of the occurrence of renal enlargement with injections of proteins but not with injections of protein hydrolysate or urea, which must be considered, is that the renal enlargement might have been caused at least in part by effects on the kidney of protein molecules *per se*, perhaps more specifically by effects on the tubular cells of the increased amount of protein filtered through the glomeruli, rather than entirely by effects of products of protein digestion and metabolism reaching the kidney through the blood stream. It might be pointed out that an elevation in urinary protein excretion has been observed incidentally accompanying an increase in the dietary protein level in rats (30, 31); however, it is not intended to suggest that the increase in proteinuria was the cause of the renal hypertrophy which occurred with the high protein diets. It is difficult to understand why the protein hydrolysate injections in the present experiments did not induce some increase in proteinuria and renal enlargement through the same mechanisms as were involved with increasing the protein content of the diet.

Morphological observations by a number of European investigators, and

more recent studies by Oliver (21), Smetana and Johnson (32), Smetana (33), and Rather (34), have provided fairly conclusive evidence that at least certain proteins present in the fluid passing through the tubules may be reabsorbed by the tubular cells and accumulate to some extent within these cells. However, the results of Bott and Richards (20) indicated that not more than a small portion of the filtered protein was reabsorbed under the conditions of their experiments. The reabsorbed protein under normal conditions presumably is digested by the tubular cells (34). The observations of others on protein reabsorption, together with our own morphological observations, particularly with gelatin which was the only protein seeming to produce recognizable enlargement of the tubular cells, suggested that the renal enlargement might have been caused in some part by enlargement of individual tubular cells as a result of reabsorption and temporary accumulation within the cells of protein passing through the glomeruli, together with water associated with it. The cytoplasmic changes produced by gelatin were similar to those described by Popper (35) and Skinsnes (36) in human kidneys following gelatin administration. Furthermore, an increase in metabolic activity of the renal cells associated with an increased reabsorption and degradation of protein might have been responsible for some degree of enlargement of the kidneys.

An increase in volume of intraluminal material within the renal tubules very probably was present in a few animals in which severe renal injury occurred, and in some other gelatin-treated animals particularly during the early periods of the injections, but it appeared unlikely that changes of this nature were responsible for the renal enlargement which regularly followed the protein injections.

In the majority of animals receiving each of the 3 per cent protein solutions, there was no evidence of gross disturbances of renal function. Renal clearance studies have not been done, though such studies might yield interesting results. The changes in renal size and the alterations in the appearance of the convoluted tubules were regarded for the greatest part as reversible morphological expressions of exaggerated physiological processes, and no inflammatory, necrotic, or sclerotic changes were observed in the kidneys, either during or following the injections. Urinary protein excretion promptly returned to normal levels after injections were discontinued. It was concluded that under the conditions of the experiments, prolonged continuous proteinuria of the degree obtained with these injections in most cases did not lead to a persistent increase in glomerular permeability or to any other form of chronic or progressive renal injury.

Definite evidences of tubular injury of a severe degree were observed, however, in a few animals receiving 3 per cent gelatin, and dilatation of glomerular capsules and tubules was present in some of the animals of series 9 receiving gelatin. Whereas most of the gelatin deposition presumably occurred in the tubular cells in the outer two-thirds of the cortex, the zone of injury was about

the corticomedullary junction. Whether this injury was a result of plugging of the tubules, or was due to some other action of gelatin, or was unrelated to any effects of gelatin on the kidney, was not determined with certainty. While the destruction of cells and proliferative changes in the dilated tubules might be considered evidence against mechanical plugging alone as the cause of the tubular lesions, at least the damaged tubules in most cases contained more protein within the lumina than usually was observed in undamaged tubules. Urine from the animals in which severe injury occurred with gelatin, unfortunately was not collected or examined. The possibility that these animals became dehydrated, due to failure to absorb part of the injected fluid, or due to failure for some reason to drink water after the injections were discontinued, in the case of the animals of series 4 which were not autopsied for several days after injection, and that dehydration contributed to the production of the injury,—as has been observed with hemoglobin (37),—must be considered. It should be noted that the concentration of protein in the urine of most of the animals in the present experiments, particularly those receiving albumin and globulin, and those of the first group receiving serum, was not as great as is often observed clinically. Furthermore, in animals with already diseased kidneys, the effects of protein injections and increased proteinuria might have been quite different from the effects observed in the present experiments employing animals with normal kidneys.

Before examining the kidneys of the second series of rats receiving homologous serum, it was considered likely that the erratic absorption of serum, and the oliguria or anuria which occurred, and perhaps also the large amounts of protein in the urine, were due to shock rather than to primary renal damage. However, the remarkable lesions which were observed were not similar to any lesions which have been attributed to disturbances of the renal circulation accompanying shock. Tubular plugging may have played a part in the production of the lesions; both the tubules and the glomerular capsules often contained large amounts of protein. The lesions were similar in certain respects to those which occurred in some of the rats receiving gelatin. Because of the great difference in the results obtained in the first and second experiments with rat serum, the profound anoxia to which the donor animals in the second experiment were subjected warrants further investigation as the possible cause of the noxious effects of the serum. It may be that the injury was due to bacterial or chemical contamination of the serum or changes in the serum subsequent to collection, and, until an attempt has been made to repeat the results, no conclusions concerning the etiology of the lesions are justified.

Finally, it might be noted that the kidneys of the protein-treated animals of the present experiments, particularly those receiving gelatin, bore certain morphological resemblances to "nephrotic" kidneys. These similarities in appearance suggested the *possibility* that, as has been concluded by a number

of investigators, certain of the changes which are seen in kidneys in diseases characterized by high levels of protein in the urine may be secondary alterations due to excessive amounts of protein passed through the glomerular membranes. Obviously no conclusions regarding the nature or the site of the fundamental disturbances in nephrosis can be drawn from these observations.

SUMMARY

Repeated intraperitoneal injections twice daily of various proteins into young rats were regularly accompanied by an increase in the protein content of the urine, significant renal enlargement, and often some degree of renal pallor. The most marked changes were induced by gelatin, followed in order by human albumin and bovine globulin. Rat serum produced similar but less conclusive changes. Similar changes were not produced by equivalent amounts of urea or casein hydrolysate.

In sections from the kidneys of animals receiving gelatin, the cells of the convoluted tubules appeared enlarged, and they contained clear "spaces" throughout the cytoplasm. The tubular cells of the animals receiving the other solutions were not obviously altered in size or shape, and the cytoplasmic changes were slight or absent. There was little evidence of increased multiplication of cells or of tubular dilatation in the kidneys of any of the groups.

Changes in concentrations of plasma proteins and hemoglobin, and the results of preliminary studies of the injected proteins in urine and renal tissue following the injections, are described and their possible significance discussed.

It appears that the renal enlargement, as well as the increase in proteinuria and the tubular alterations which followed the protein injections, might have been caused in part by effects on the kidney of protein molecules *per se*, perhaps most likely by the effects on the tubular cells of an increased amount of protein filtered through the glomerular membranes, rather than entirely by effects of products of protein digestion and metabolism reaching the kidney through the blood stream.

In the majority of animals there was no evidence from the morphological or functional studies, that the prolonged and continuous proteinuria induced by the protein injections resulted in renal damage, unless the renal enlargement, and the cytoplasmic changes which occurred regularly with gelatin, are considered evidence of damage. Renal enlargement and proteinuria promptly regressed after injections were discontinued.

Lesions characterized by severe degrees of tubular damage, possibly as a result of tubular plugging, were observed in some of the animals of one group receiving gelatin solution of the usual concentration, and dilatation of renal tubules and glomerular capsules was present in some other gelatin-treated animals autopsied after relatively brief injection periods. A description is also presented of lesions

of remarkable character which developed in the kidneys of all the animals of one small group receiving homologous serum obtained from severely anoxic donors.

The possible relationship between the renal changes in the protein-injected animals and certain alterations of the kidneys observed in diseases characterized by large amounts of protein in the urine, is considered.

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EXPLANATION OF PLATES

PLATE 32

The photographs were made by Mr. R. F. Carter.

FIG. 1. Comparing left kidney from gelatin-treated rat (above) with homolateral kidney from comparable saline-injected control animal (below). The organs had not been fixed. The animals were from series 1 and received injections for 6 weeks. Note enlargement, particularly in the cortical portion, and paleness of the kidney from the gelatin-injected animal. Weights are as follows:—

$$\text{Control rat: weight of kidneys, 1.85 gm.; weight of rat, 146 gm.; } \frac{\text{Weight of kidneys}}{\text{Weight of rat}} \\ \times 100 = 1.27$$

$$\text{Gelatin rat: weight of kidneys, 2.50 gm.; weight of rat, 140 gm.; } \frac{\text{Weight of kidneys}}{\text{Weight of rat}} \\ \times 100 = 1.79$$

Magnification $\times 2$.

FIG. 2. Comparing left kidney from albumin-treated rat (above) with control kidney (below). The animals were from series 2, and received injections for 5 weeks. Note enlargement and cortical thickening in kidney of albumin-treated animal. Weights are as follows:—

$$\text{Control rat: weight of kidneys, 1.54 gm.; weight of rat, 126 gm.; } \frac{\text{Weight of kidneys}}{\text{Weight of rat}} \\ \times 100 = 1.22$$

$$\text{Albumin rat: weight of kidneys, 2.10 gm.; weight of rat, 126 gm.; } \frac{\text{Weight of kidneys}}{\text{Weight of rat}} \\ \times 100 = 1.67$$

Magnification $\times 1.5$.

FIG. 3. Comparing right kidneys from comparable rats of series 6 receiving various solutions. Animals received injections for 9 days. Top row, left to right:

$$\text{Gelatin: weight of kidneys, 1.10 gm.; weight of rat, 64 gm.; } \frac{\text{Weight of kidneys}}{\text{Weight of rat}} \\ \times 100 = 1.72$$

$$\text{Albumin: weight of kidneys, 1.03 gm.; weight of rat, 66 gm.; } \frac{\text{Weight of kidneys}}{\text{Weight of rat}} \\ \times 100 = 1.56$$

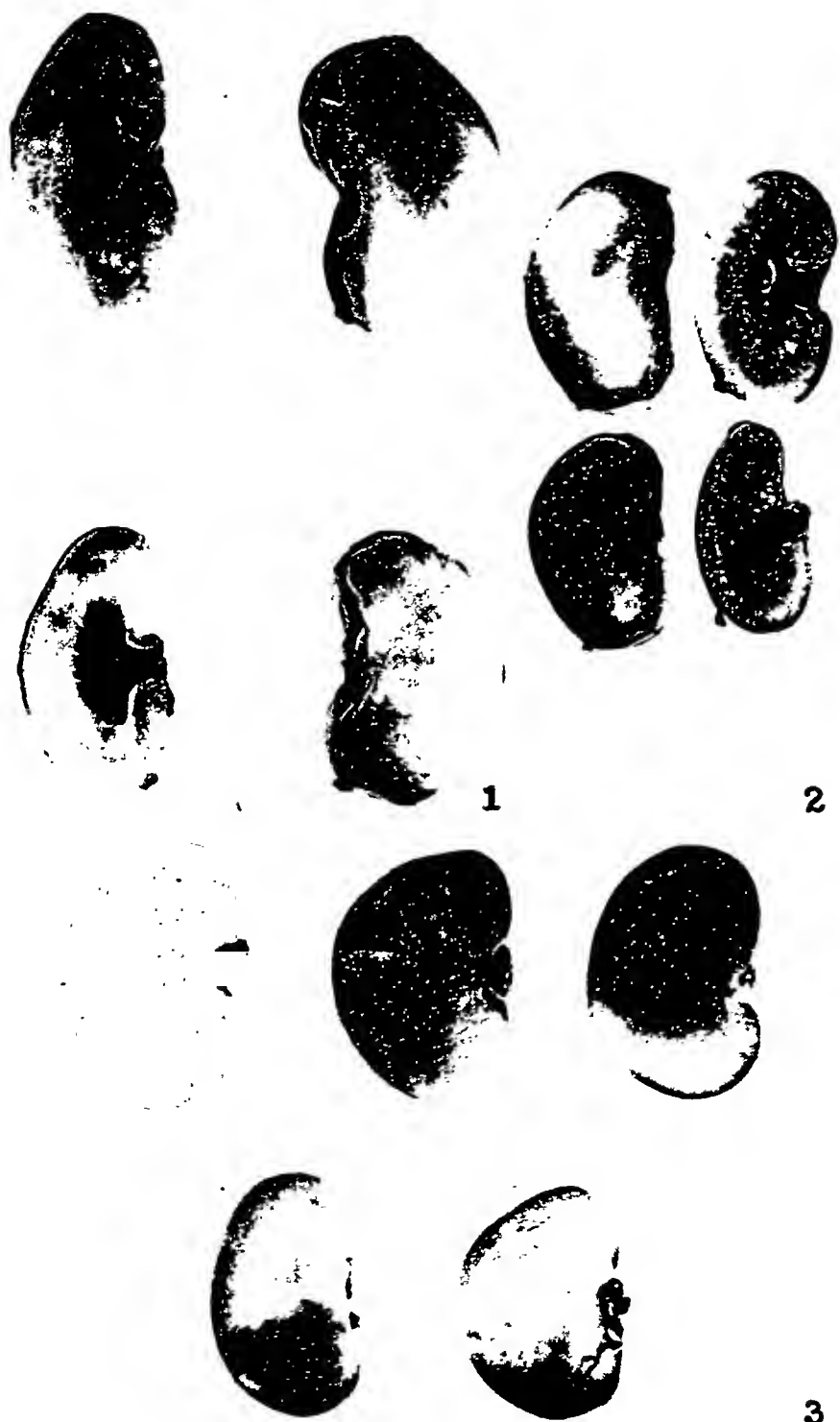
$$\text{Globulin: weight of kidneys, 1.03 gm.; weight of rat, 66 gm.; } \frac{\text{Weight of kidneys}}{\text{Weight of rat}} \\ \times 100 = 1.56$$

Bottom row, left to right:

$$\text{Casein hydrolysate: weight of kidneys, 0.78 gm.; weight of rat, 64 gm.; } \frac{\text{Weight of kidneys}}{\text{Weight of rat}} \times 100 = 1.22$$

$$\text{Saline: weight of kidneys, 0.78 gm.; weight of rat, 64 gm.; } \frac{\text{Weight of kidneys}}{\text{Weight of rat}} \\ \times 100 = 1.22$$

Magnification $\times 4$.



(Baxter and Cotzias: Parenteral protein and the kidney)

PLATE 33

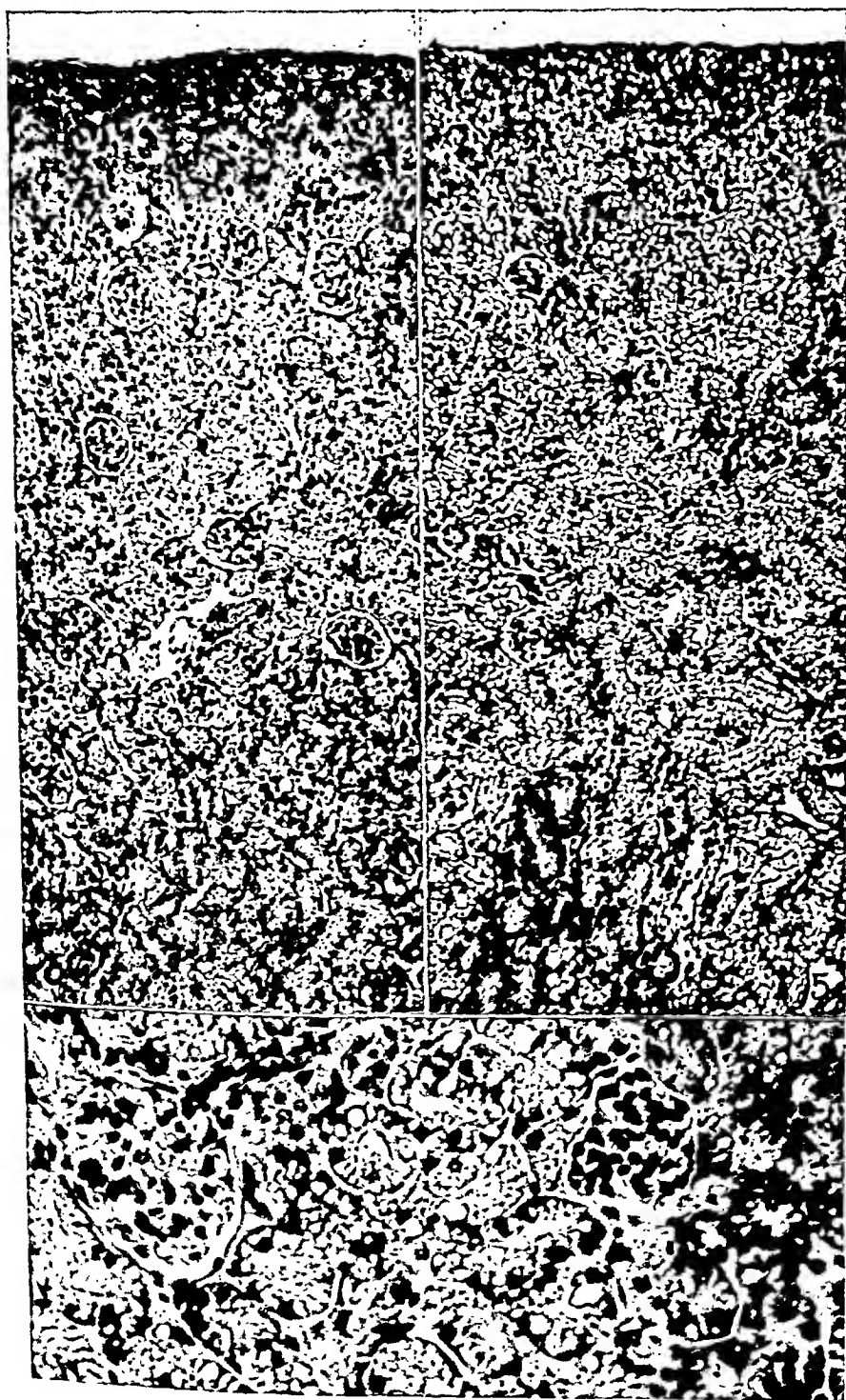
All the sections were stained with hematoxylin and eosin.

The photographs were made by Mr. J. A. Carlile.

FIG. 4. Sagittal section through a control kidney of same group as one shown in Fig. 1. $\times 120$.

FIG. 5. Similar section through kidney of comparable gelatin-treated animal of series 1. Note enlargement of cells of convoluted tubules, and what appear to be clear spaces within the cytoplasm of the cells. $\times 120$.

FIG. 6. Higher magnification of section shown in Fig. 5, showing cytoplasmic alterations in greater detail. $\times 350$.



(Baxter and Cotzias: Parenteral protein and the kidney)

PLATE 34

All the sections were stained with hematoxylin and eosin.

The photographs were made by Mr. C. F. Reather, Johns Hopkins School of Medicine.

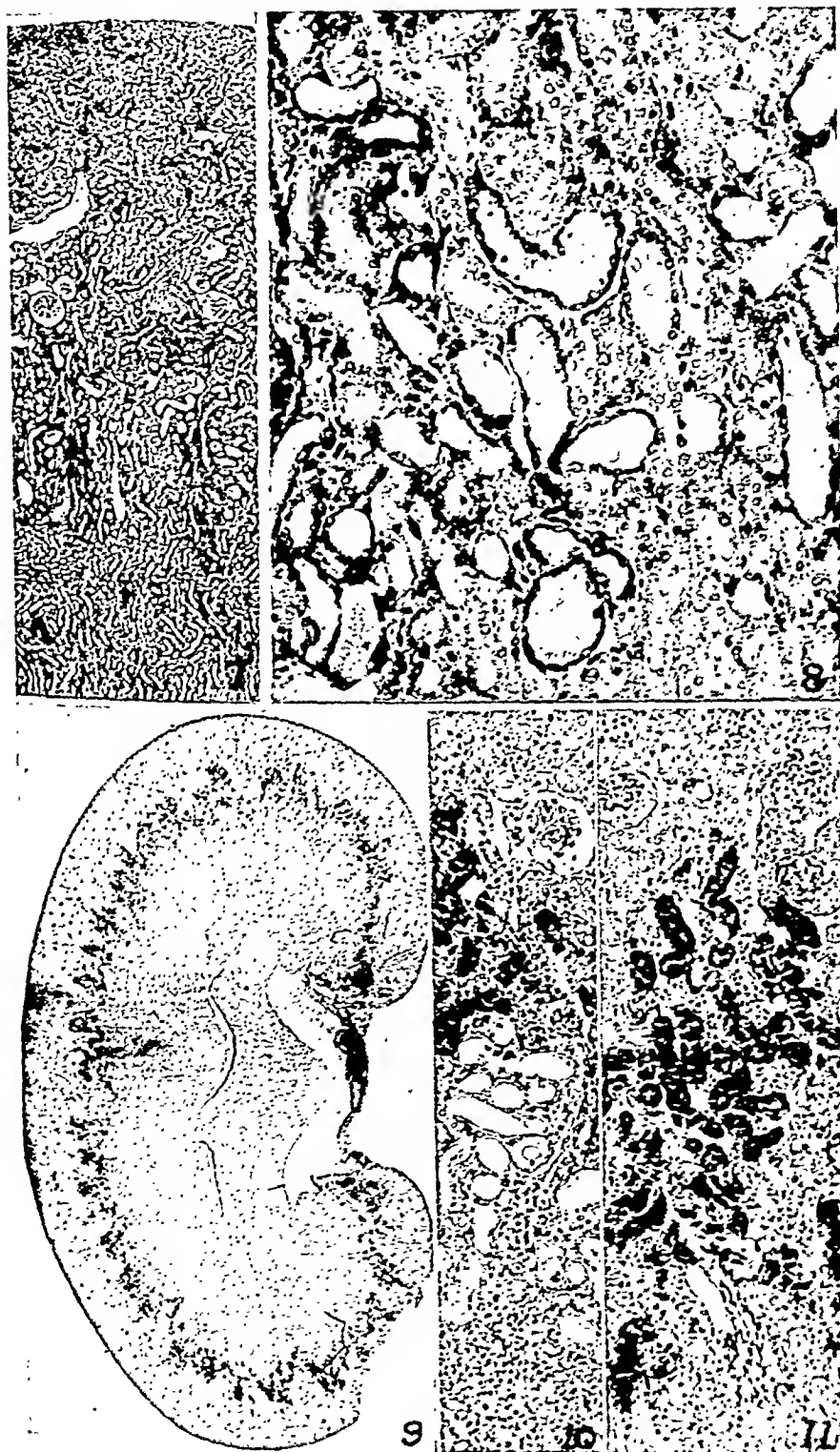
FIG. 7. Sagittal section through kidney of gelatin-treated animal of series 4. This animal received only three injections of gelatin at 12 hour intervals, and was autopsied 54 hours after the last injection. At a higher magnification clear spaces could still be seen in the cells of the convoluted tubules in the outer two-thirds of the cortex. Deeper in the cortex and in the outer portion of the medulla, many glomerular capsules and tubules are dilated. This section shows the most extensive damage observed in any of the kidneys of the animals receiving the 3 per cent solution of gelatin. $\times 40$.

FIG. 8. Higher magnification of the section shown in Fig. 7, showing details of the tubular alterations in the zone of the most extensive injury. Many of the tubules are dilated, lined by flattened and markedly basophilic epithelium, and contain pink-staining protein material. Mitotic figures and other nuclear changes indicative of active cellular proliferation were also present. $\times 200$.

FIG. 9. Sagittal section from kidney of a serum-treated animal of series 8, which became obviously ill and was autopsied on the 5th day. This animal was anuric through a considerable part of the injection period but excreted a moderate amount of urine containing large amounts of protein during the 24 hours before autopsy. The blood urea level was approximately 100 mg. per cent at the time of autopsy. The zone of tubular necrosis and calcification, and the zone of tubular dilatation, are shown well. $\times 8$.

FIG. 10. Higher magnification of section shown in Fig. 9. The zone of calcification can be seen about the region of the corticomedullary junction. Adjacent to and toward the pelvis from this calcified zone, there is the zone of dilated tubules, many of which contain protein. Many tubules far down toward pelvis, which were not dilated, also were full of protein material. $\times 100$.

FIG. 11. A similar section from kidney of the other serum-treated animal of series 8 which was autopsied on the 5th day. In this kidney most of the glomerular capsules contained protein precipitates. $\times 100$.



(Baxter and Cotzias: Parenteral protein and the kidney)

SEROLOGICAL STUDIES IN RHEUMATIC FEVER

I. THE "PHASE" REACTION AND THE DETECTION OF AUTOANTIBODIES IN THE RHEUMATIC STATE

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It has been suggested that an allergic type of mechanism may be responsible for the rheumatic process. However, it must be recognized that the evidence for this is far from conclusive. The allergic hypothesis of the disease is based chiefly upon certain characteristics of the disease which suggest allergy (5) such as the latent period after infection with *S. hemolyticus* (1-5), the clinical similarity to serum sickness (6), and the morphological analogy between rheumatic lesions and those produced by necrotizing allergic reactions in experimental animals (7-11). It is generally recognized that infection with the hemolytic streptococcus is frequently followed by the appearance of circulating antibodies (12, 13) or skin sensitivity (14-16) to streptococcus products. However, these responses do not appear to differ qualitatively or quantitatively in rheumatic subjects from those of non-rheumatic individuals with streptococcus infections, particularly if the infection is persistent (17-19). Identification of the antigens or antibodies involved in the presumed allergic reaction in rheumatic fever would give experimental support to the allergic hypothesis. There are relatively few reported studies concerned with the demonstration of antibodies in rheumatic patients which are not found in other individuals recovering from streptococcus infections. Thus, the phase reaction reported by Coburn and Pauli (20), and the development of autoantibodies to liver tissue (Brokman, Brill, and Freundel (28)) and to heart tissue (Cavelti (30)) have been advanced as serological phenomena peculiar to the rheumatic state, and possibly related to the necrotizing allergic reaction presumed to occur. The present study is concerned with an attempt to repeat and, if possible, to extend previous investigations indicating the existence of allergic mechanisms in rheumatic fever.

A. The Phase Reaction

In 1939 Coburn and Pauli (20) reported that a substance called a "precipitinogen" appeared in the serum of a rheumatic subject following a sore throat which precipi-

* This work was done during the tenure of a Life Insurance Medical Research Fellowship. Aided in part by the Masonic Foundation for Medical Research and Human Welfare.

tated when mixed with the serum taken during the subsequent rheumatic attack. Because the reaction occurred with serum obtained during phase I or II (the sore throat and the latent periods), when mixed with serum from phase III (the period of rheumatic activity), it has been called the "phase reaction." If a rheumatic attack did not develop following the sore throat, it was reported that the precipitinogen was not present, and no precipitin developed. When the rheumatic attack ran a polycyclic course, the precipitinogen and the precipitin alternately appeared in the serum. It was suggested that this precipitinogen might represent a "secondary antigen" derived from a combination of streptococcal products and human tissue constituents. Although the phenomenon did not necessarily represent an antigen-antibody reaction, it appeared to be one, and one intimately associated with the occurrence of rheumatic fever.

Despite the apparently fundamental significance of this reaction, there were no further reports concerning it until 1946 when Wedum and Wedum (21) seemed to have confirmed some of the observations of Coburn and Pauli. However, there are certain differences between the two reports which would appear to be incompatible with the hypothesis suggested by both groups of investigators. Wedum and Wedum found that their presumed antigen occurred during the first few days *after* the onset of a rheumatic attack, not preceding it, and again appeared at the end of the attack in contrast to Coburn's observations. Occasionally both substances, *i.e.* precipitinogen and precipitin, were said to coexist in the same serum sample, because a fine precipitate occurred when the unmixed sample of serum was incubated (21). Wedum and Wedum also found that the phenomenon was not peculiar to rheumatic fever. It occurred in uncomplicated nasopharyngitis, in primary atypical pneumonia, and in a few miscellaneous conditions. Unfortunately, much of this work was done with the sera of different individuals; that is, serum from one subject with a sore throat was mixed with that of another subject who had rheumatic fever.

Experimental.—Sera were obtained from patients on the wards and in the Rheumatic Fever Follow-up Clinic of the Presbyterian Hospital, the Babies Hospital, and The Pelham Home for Children. The latter institution, a convalescent home, offered an opportunity to obtain sera from known rheumatic subjects before the onset of a sore throat or of a rheumatic recrudescence. Sera were obtained at weekly intervals, generally more frequently during active illness, and less frequently during late convalescence and quiescent periods. Usually sera taken at different times from the same individual were used for one series of tests. In a number of instances sera from these patients were intermixed with no alteration in results.

The tests were performed using the technique previously described (20). 0.1 ml. of each serum to be tested was mixed with an equal quantity of another sample from the same patient. The mixture was incubated at 37°C. for 2 hours, and put in the ice box overnight. After centrifugation the next morning, tubes were agitated and examined with a lens. Particulate matter, scarcely visible to the unaided eye, was the strongest positive reaction obtained, as in the original work. Readings were graded as \pm , +, and ++, and independent readings by each author gave little significant variation. Unlike the previous work, controls of test serum alone, and test serum plus normal serum, were used in addition to the serum plus saline controls. Every serum was cross-tested with every other serum from the same patient.

The sera tested included 221 samples from 38 cases of rheumatic fever (20 cases with only a single serum sample); 16 samples from 7 cases of streptococcus pharyngitis in non-rheumatic individuals; 85 sera from 11 cases of streptococcus pharyngitis in rheumatic subjects who did

not develop demonstrable rheumatic activity; and 21 sera from 4 miscellaneous cases—2 of "idiopathic" myocarditis, 1 of acute gonorrheal arthritis, and 1 of an asymptomatic inactive rheumatic subject.

The results of the tests are shown in Table I. From 18 active rheumatic patients followed for long periods of time (Table I, A), negative, irregularly positive, and uniformly positive precipitin reactions were obtained. When positive precipitin tests were obtained they were obtained with any combination of serum samples irrespective of their relationship to the phase of the disease. Indeed, many of these positively precipitating sera also showed positive reactions when mixed with control non-rheumatic sera. Table I, A illustrates the haphazard relationship of a negative or a positive precipitation test to the periods of sore throat, and to the course of active rheumatic fever. Table I, B and I, C presents the results of the "phase reaction" test in inactive rheumatic subjects and in normal (non-rheumatic) patients with sore throats not followed by attacks of rheumatic fever. Of the inactive rheumatic group (Table I, B) positive precipitation was noted with the sera from four patients, no precipitation occurred with the sera from four other patients; and doubtfully positive precipitation was noted with the sera from the remaining three individuals. There is a preponderance of negative precipitation tests in the non-rheumatic group with sore throats (Table I, C). It should be noted that fewer sera were obtained in these instances. Samples of serum from a case of gonococcal arthritis (Table I, D) were positive throughout. A completely inactive and asymptomatic subject with a history of rheumatic fever 3 years previously gave scattered weakly positive and doubtfully positive precipitation reactions. Therefore, no correlation was found between the occurrence of precipitates and the occurrence of any clinical episode, either sore throat or rheumatic fever. Furthermore, repeated tests failed to give reproducible results.

Further evidence against a possible antigen-antibody reaction occurring in these sera appeared from the following observations:

When one or both sera usually showing precipitates were diluted with an equal amount of saline, and mixed, no precipitation occurred. If either serum contained a true precipitin, and the other an antigen, slight dilution of one or the other of the reagents would not be expected to affect the formation of the precipitate.

Sera, showing precipitate, were mixed in various dilutions in the presence of complement. No fixation of complement occurred if amounts of serum below the anticomplementary concentrations were used. Certain known antigen-antibody systems do not fix complement. Therefore, this observation is only suggestive of the absence of an immune system.

Carmine dye particles (22) and collodion particles (32) were used in an attempt to make a microscopic reaction more visible. No agglutination of the mixtures occurred.

Serum obtained from one patient after a sore throat was injected intracutaneously during his rheumatic attack. No skin reaction occurred, either of the immediate or delayed type. Similarly an attempted Prausnitz-Küstner reaction in a normal individual with the sera containing the "precipitinogen" and "precipitin" gave negative results.

TABLE I
The Results of Precipitin Tests for the "Phase Reaction"

Patient	No. of sera	Periods during which sera were obtained	Results and comments on cross-precipitin tests in relation to course	Precipitin reaction with normal sera of same blood group
<i>A. Acute Rheumatic Fever</i>				
J. M.	18	(a) Initial sore throat. (b) Latent period, 1 mo. (c) Polycyclic rheumatic fever for 4 mos.	Slight precipitation scattered throughout	+
J. B.	11	(a) Early and polycyclic acute rheumatic fever (b) Quiescent convalescence over 4 mos.	Precipitation present in all tests	+
F. M.	6	(a) Very early acute rheumatic fever for 2 wks. (b) Quiescent convalescence for 3 wks.	Two sera at end of period of activity showed weak precipitation with each other and when alone. All others negative	-
A. T.	16	(a) Latent period 3 wks. (b) Early rheumatic fever. (c) Polycyclic course for 4 mos.	Scattered precipitation, particularly with one of latent period sera against all later sera and against controls	+
R. C.	3	(a) Acute rheumatic fever becoming normal by all usual criteria in 10 days	Weak precipitation between 1st and 3rd sera	-
B. V.	15	Acute rheumatic fever polycyclic for 3 mos.	All negative	-
A. G.	4	(a) Acute rheumatic fever for 1 mo. (b) Convalescent 1 mo.	First serum precipitates with remaining three but all four precipitate with control	+
C. D.	3	Acute rheumatic fever, 1 mo. duration	All sera form slight precipitates with each other, by themselves, and with controls	+
G. W.	4	(a) Acute rheumatic fever for 1 mo. (b) Quiescent for 1 mo.	Scattered precipitates in six of the tests but three are sera alone	+

TABLE I—Continued

Patient	No. of sera	Periods during which sera were obtained	Results and comments on cross-precipitin tests in relation to course	Precipitin reaction with normal sera of same blood group
<i>A. Acute Rheumatic Fever—Continued</i>				
W. H.	3	(a) Acute rheumatic fever for 2 wks. (b) Quiescent in 3rd wk.	All negative	—
R. A.	6	(a) Sore throat (b) Latent period for 2 wks. (c) Asymptomatic exacerbation for 4 wks. (ESR rising to 80)	All sera precipitate against each other	—
B. D.	19	(a) Asymptomatic 2 wks. (b) Sore throat 1 wk. (c) Latent period 3 wks. (d) Exacerbation 5 wks. (e) Quiescent 9 wks.	Scattered precipitations unrelated to course	+
H. H.	17	(a) Sore throat 1 wk. (b) Latent period 2 wks. (c) Active rheumatic fever 13 wks. polycyclic (d) Quiescent 5 wks.	Scattered weak precipitations—no relation to course	+
M. P.	18	(a) Sore throat 1 wk. (b) Latent period for 5 wks. (c) Acute rheumatic fever for 12 wks. (d) Quiescent for 6 wks.	As above	+
A. S.	24	(a) Asymptomatic 3 wks. (b) Tonsillitis 1 wk. (c) Immediate onset acute rheumatic fever which continued polycyclic for 15 wks. (d) Quiescent 4 wks. (e) ESR rising to 43 for 3 wks., then normal (f) 4 samples in next 4 mos.	As above	—
G. K.	13	(a) Subsiding acute rheumatic fever (b) Quiescent 2 wks. (c) Sore throat 1 wk. (d) Asymptomatic 3 wks. (e) Recurrent activity 3 wks. (f) Quiescent for 2 mos.	All tests showed weak precipitates	+

TABLE I—Continued

Patient	No. of sera	Periods during which sera were obtained	Results and comments on cross-precipitin tests in relation to course	Precipitin reaction with normal sera of same blood group
<i>A. Acute Rheumatic Fever—Continued</i>				
E. C.	11	(a) Subsiding acute rheumatic fever 6 wks. (b) Quiescent 14 wks.	As above	+
B. F.	17	(a) Rheumatic fever for 3 mos. (b) Quiescent for 1 mo. (c) Chorea for 1 mo.	Scattered positives with no relation to course	+
6 other cases of acute rheumatic fever—sera obtained at various times during the course—all gave a weak precipitate alone, with one another and with controls				+
<i>B. Inactive Rheumatic Subjects with Sore Throats Not Followed by Recrudescences</i>				
R. B.	7	Sore throat for 4 days. Elevated ESR for 2 wks. Quiescent for 6 wks.	All sera precipitate weakly with each other and with controls	+
I. R.	4	Sore throat	All negative	—
C. V.	3	Sore throat—followed for 2 wks. after	All negative	—
A. I.	4	Sore throat 1 mo. thereafter	All negative	±
C. S.	14	Followed 4 mos. at convalescent home, before, during, and after sore throat without rheumatic activity	Scattered precipitates unrelated to any period of observation	Occasionally positive
R. H.	10	5 mos. at convalescent home. One sore throat. No active rheumatic fever	All tests show precipitate formation	+
A. H.	9	As above	Scattered precipitates	+
L. L.	11	As above	All doubtfully positive	±
B. C.	6	As above	All doubtfully positive	+
P. C.	8	As above	All doubtfully positive	+
S. C.	9	As above but with two sore throats	All tests negative	—

TABLE I—*Concluded*

Patient	No. of sera	Periods during which sera were obtained	Results and comments on cross-precipitin tests in relation to course	Precipitin reaction with normal sera of same blood group
<i>C. Non-Rheumatic Subjects with Streptococcus Pharyngitis</i>				
G. D.	2	(a) Sore throat (b) Quiescent 2 wks. later	Doubtful precipitation	—
G. G.	2	Same but 18 days apart	Negative	—
H. H.	2	Same 12 days apart	Negative	—
B. G.	3	Sore throat, 5 days and 1 mo. later	Negative	—
T. M.	2	2 days apart	Negative	—
M. O'C.	2	13 days apart	Negative	—
R. S.	3	Sore throat, 3 days and 13 days later	Negative	—
<i>D. Miscellaneous Cases</i>				
J. R.	8	Old rheumatic subject. Asymptomatic and quiescent for 3 mos.	Scattered weak and doubtful precipitates	±
R. T.	3	3 wks. of gonorrheal arthritis	All tests precipitate including controls	+
W. M.	5	"Idiopathic" myocarditis 5 wks.	All tests negative	—
B. S.	5	Probable "idiopathic" myocarditis and pulmonary embolism 6 wks.	All tests negative	—

Serum of a patient which did show a tendency to form particulate matter with other sera, including control sera, showed the same tendency, if after incubation by itself, and centrifugation, the supernatant was again incubated and centrifuged. Therefore, the "reagents" could not be absorbed from the sera.

Discussion.—The "phase reaction," a serological phenomenon thought to be specific in rheumatic individuals has been cited as laboratory evidence for the hypothesis that an allergic mechanism is concerned with the development of rheumatic fever. This study has not corroborated previous reports. The inconstant nature of the formation of precipitates on repeating identical tests,

and the lack of visible formation of precipitates on dilution with saline, suggest that the particulate matter formed in these tests is not due to a specific immune reaction. It is perhaps not unlike the non-specific precipitation that occurs in uncontaminated sera which are allowed to stand for a period of time, even in the ice box. Sera cleared of particulate matter after prolonged centrifugation in the cold before the performance of a quantitative precipitin test (23) occasionally develop particulate matter after incubation at 37°C. This may result in a precipitate giving an appreciable amount of nitrogen in the serum control or "blank" tubes. There are a number of poorly understood changes in the sera of patients with rheumatic fever and certain other illnesses which are reflected in phenomena such as the erythrocyte sedimentation rate (25) and the "colloidal" tests (26). In addition, chemical changes are known to occur in active rheumatic sera (24, 27). These abnormalities may or may not be related to the non-specific precipitation that occurs in serum when it stands alone in the ice box or when it is mixed with another serum and incubated.

B. Autoantibodies in Rheumatic Fever

Autoantibodies and isoantibodies occasionally cause severe necrotizing allergic reactions, as in the Donath-Landsteiner reaction, erythroblastosis foetalis and, occasionally, cold hemagglutination. However, such antibodies may exist without obvious widespread tissue damage, as in the case of the Wassermann antibody. Therefore, the demonstration of an autoantibody in rheumatic fever may be of interest but does not *per se* establish the rôle of the tissue reaction in response to the autoantibody in rheumatic fever. The concept of an autoantibody in rheumatic fever was suggested by Brokman, Brill, and Freundzel (28). They found that sera from rheumatic patients fixed complement when mixed with an extract of liver obtained at the autopsy of a rheumatic child. Sera from other diseases did not fix complement with this "antigen." Unfortunately, anticomplementary controls were not reported. It is well known that tissue extracts are frequently anticomplementary, as are, occasionally, rheumatic or other sera in certain concentrations. Furthermore, Eaton and his associates reported that complement fixation occurs when liver tissue is mixed with sera from a variety of illnesses as well as with some normal sera (29).

Experimental.—Tests were made for a reaction similar to that of Brokman, Brill, and Freundzel. In the absence of fresh liver tissue, heart and lung tissue from a rheumatic individual, and placental tissue from a living rheumatic subject were used as "antigens." Eight sera, from active rheumatic subjects of the same blood groups as the individuals from whom the tissues had been obtained were mixed in various dilutions in the presence of 4 hemolytic units of complement. The traditional technique was employed, with 0.2 ml. of diluted "antigen," 0.2 ml. of diluted serum, and 0.2 ml. of complement. After incubation for a half hour, 0.2 ml. of a standardized suspension of sensitized sheep cells was added. No evidence of complement fixation was found in dilutions of serum and of tissue extract which were not anticomplementary alone.

Another autoantibody system was suggested by Cavelti (30). He found that one of four normal (*i.e.*, non-rheumatic) hearts used as antigen reacted strongly with sera from 27 of 36 rheumatics studied. By mixing the antigen with collodion particles, the presumed immune aggregation was made macro-

TABLE II

Results of the Collodion Particle Method for the Detection of "Autoantibodies" in the Sera of Rheumatic and Syphilitic Patients

Patient and serum No.	Antigens														
	M. (carcinoma)					W. (chronic nephritis)					H. (rheumatic)				
	Heart	Lung	Liver	Spleen	Kidney	Heart	Lung	Liver	Spleen	Kidney	Heart	Lung	Muscle	Spleen	Kidney
Rheumatic fever															
Zi. 1	0	+	+	0	0	0	0	0	0	0	0	+	0	0	0
" 2*	0	0	++	++	+	0	0	0	0	0	0	0	0	0	0
Pe. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	++	0
" 2	0	0	0	0	0	0	0	0	0	0	0	0	0	++	0
Ki. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	++	0
" 2	0	0	0	0	0	0	0	0	0	0	0	0	0	++	0
Va. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
" 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dr. 1	±	0	±	0	0	0	0	0	0	0	0	0	0	0	0
" 2	0	0	0	++	0	0	0	0	0	0	0	0	0	0	0
Du. 1	0	0	0	0	0	0	0	0	0	0	0	+	0	0	0
To. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
We. 1	0	0	0	0	0	0	0	0	0	0	0	+	0	0	0
Fe. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mc. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pi. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
" 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Te. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
" 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
An. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
" 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Wi. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ma. 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
" 2	0	0	0	0	0	0	0	+	0	0	0	0	0	0	0
Syphilis															
A	0	0	0	0	0	0	0	0	0	0	0	0	0	+++	+++
B	0	0	0	0	±	0	0	0	+	0	0	0	0	0	0
C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D	0	0	0	±	++	0	0	0	+	0	0	0	0	0	0

* Serum 2 is at a later period in the course of the disease.

scopically visible. When the surface of the particles is coated with antigen, the whole particle exhibits many of the properties of its protein covering (31). A reaction of antigen on the particle surface with its antibody results in macroscopic agglutination (32).

Experimental.—Colloidal particles were prepared by the method of Cavelti (33). To tubes containing 0.2 ml. of a mixture of collodion particles and a dilution of a clear saline extract of

the antigen, 0.5 ml. of antiserum in various dilutions was added. After being mixed and standing at room temperature for 2 hours, the mixture was centrifuged, agitated, and the degree of agglutination was observed.

Tests were made to determine the optimal concentration of particles, antigen, and antibody, using two known systems: rat kidney and rabbit anti-rat kidney serum R 673 (obtained from Dr. B. C. Seegal), and the type-specific polysaccharide SS II of the Type II pneumococcus (obtained from Dr. M. Heidelberger) and its homologous rabbit antibody. Although the collodion particle method appeared to give positive results with known antigen-antibody systems, inconstant quantitative relationships were obtained at various times when the same system was repeated. No false positive results were obtained with good preparations of collodion particles.

Five or more tissues were obtained within 12 to 24 hours after the death of three individuals. One case (H.) had inactive rheumatic heart disease and pulmonary edema. The other two cases had carcinoma of the gall bladder (M.) and chronic glomerulonephritis (W.). In addition, heart tissue from one active rheumatic individual, and tonsil specimens from two inactive rheumatic children were also obtained. The tissues were kept in the solid CO₂ storage chest until ready for use. They were then ground with sand and extracted with cold saline to make a 20 per cent mixture by weight. After centrifugation, the clear supernate was separated for use in the tests. In all instances the tissues were from individuals of blood group O. All the tests reported employed group O sera to obviate the possibility of a false positive test due to an incompatible blood group reaction. For control tests, normal sera and uncoated normal particles were used, as well as the variety of tissues and concentrations. In addition, a precaution not previously employed was used. Known positive Wassermann sera from syphilis served as controls.

Several batches of particles occasionally gave a preponderance of negative or of positive results with the tissue extract-serum systems, although particles from the same lot appeared to be similar to previous batches in their reaction with the known antigen-antibody systems.

A summary of some of the tests employing the collodion particle method is seen in Table II. All the sera listed here are from active rheumatic or syphilitic patients. Normal sera rarely gave a positive reaction with one of the tissue extracts. The sera from ten of the active rheumatic patients gave no reaction, or a rare weakly positive agglutination. Five gave positive reactions, usually against more than one tissue. In other tests, not shown here, these and other sera appeared to cause most marked agglutination with extracts of tonsil, spleen, kidney, and lung. Since streptococci were cultured from the tonsils at operation, antibodies to the streptococci were probably instrumental in causing the marked agglutination of the tonsillar tissue.

Discussion.—The occurrence in rheumatic sera of agglutinins to collodion particles coated with a heart tissue extract was observed infrequently as compared with reactions to particles coated with extracts of other tissues. In addition, sera from syphilitic patients appeared to contain the agglutinins more frequently than did rheumatic sera. Since we performed these tests, Dr. Cavelti has informed us that he has had difficulty repeating his results with antigens other than the extract of the one heart tissue which gave strongly positive reactions. The diminution of his supply of the original antigen precludes any attempt at analysis. The possibility continues that the reaction noted

constitutes (1) a reaction between antibodies and bacterial contaminants of the tissues used, or (2) a modification of the flocculation tests for syphilis, since the Wassermann antigen is a constituent of normal tissues (34, 35). The occurrence of biologically false positive Wassermann reactions in many diseases has been reviewed by Davis (36).

SUMMARY AND CONCLUSIONS

1. An attempt was made to repeat and extend various tests which have been presumed to demonstrate specific antigens and antibodies in rheumatic fever.

2. The "phase reaction" appears to be an inconstant phenomenon probably related to a colloidal abnormality of the serum, rather than to a specific antigen-antibody system.

3. No specific autoantibodies to human tissue extracts were demonstrable by complement fixation or by the collodion particle technique. Variable results were noted with the same test sera on different occasions, and positive reactions with control tissues and control sera were observed.

4. The possibility should be considered that autoantibodies are not necessarily specific for rheumatic fever but may be manifestations of the occurrence of a type of reaction similar to a biologically false positive Wassermann reaction.

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THE CYTOLOGY OF RICKETTSIAE

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PLATES 35 AND 36

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In recent years it has been shown that all bacterial cells which have been adequately examined are essentially similar to the cells of higher organisms with the demonstration of desoxyribonucleic acid-containing, regularly dividing nuclear structures and the presence of ribonucleic acid in the cytoplasm (1, 2). It is not yet clear whether these Feulgen-positive bodies are similar to chromosomes in higher organisms or whether the genic material is organized in a different way. Since chromosomes exhibit a very special structure and behavior during cell division in addition to containing DNA and being self-reproducing, this name should not be applied to the Feulgen-positive bodies of bacteria. Instead the less specific terms "nuclear structure" and "chromatinic body" (Robinow (1)) will be used here.

Rickettsiae are usually considered to be essentially like bacteria in morphology though they resemble viruses in being obligate intracellular parasites (cf. reference 3). Photographs with the electron microscope have revealed some internal structures similar to those found in bacteria (4). Chemical analysis of isolated rickettsiae, however, has shown the presence of desoxyribonucleic acid only, no ribonucleic acid having been detected (5, 6). The present study was undertaken in order to investigate, first, whether RNA can be demonstrated in unwashed rickettsiae using cytochemical methods, and secondly, whether the DNA is present in nuclear structures as in the bacteria above mentioned, or is diffusely distributed through the rickettsial bodies.

Materials and Methods

The material used in this study came from chick embryo yolk sacs infected with the Breinl strain of epidemic typhus (*Rickettsia prowazekii*). Yolk sac smears were air-dried, heat-fixed, and then immersed in Carnoy or in 20 per cent formalin. Concentrated suspensions of rickettsiae were obtained from yolk sac emulsions by repeated washing in saline.

Ultraviolet photographs (2537 Å) were obtained using a G.E. germicidal lamp (4 watt) with quartz-condensing lens, a Bäckström filter (20 per cent NiSO_4 plus 8.5 per cent CoSO_4 in distilled water), Zeiss 1.7 mm. quartz objective and Zeiss $\times 10$ quartz ocular.

Rickettsiae for electron microscope photographs were extracted from yolk sacs, sulfate-precipitated, and inactivated with 1:5,000 merthiolate. A drop of this suspension was dried on formvar film, washed in distilled water to remove salts, and dried again for examination in the RCA Universal electron microscope.

Unstained smears of rickettsiae were also photographed with the phase contrast microscope (Spencer 1.8 mm., medium dark contrast objective).

To determine the presence of RNA in unwashed rickettsiae they were fixed in 20 per cent formalin and treated with ribonuclease (preparation of Dr. Kunitz, 0.2 mg. per ml. in distilled water, 45 minutes at 50°C.). Controls were treated the same way except for the enzyme. Buffer solutions were not used because they were found to extract the basophilic material from rickettsiae on the control slides. The slides were then stained together in methyl green pyronine for 20 minutes and differentiated in acetone.

Demonstration of Ribonucleic Acid in the Cytoplasm of Rickettsiae

Tovarnickij *et al.* (5) studied the chemical composition of rickettsiae isolated from mouse lungs and washed with physiological saline. Cohen (6) analyzed rickettsiae isolated from phenol-treated typhus vaccines. Both authors reported the presence of DNA, but no RNA was found. They concluded that rickettsiae were similar to viruses in containing only one type of nucleic acid, while bacteria and higher organisms always have both RNA and DNA. However, it has been shown that ribonucleoproteins are easily extracted from cells with physiological saline (7). It is therefore possible that no RNA was present in purified rickettsiae because it had been washed out during preparation. The presence of RNA in cells can be demonstrated cytochemically using ribonuclease and basic dyes (8). We therefore treated yolk sac smears fixed with 20 per cent formalin with ribonuclease and stained with methyl green pyronine. On the control slide the rickettsiae stain more or less solidly red with pyronine (Fig. 1). The intensity of the staining varies somewhat from one cell to the other. After digestion with ribonuclease, however, the over-all staining is always very much decreased (Fig. 2). Rickettsiae therefore contain RNA in variable amounts, probably depending on the physiological state as has been demonstrated for bacteria (9). Since it was not found in purified suspensions of rickettsiae it must have been lost during preparation. The effect of saline for instance on the staining with pyronine is marked. Fresh rickettsiae and rickettsiae washed with saline were smeared on the same slide and stained with pyronine. Unwashed rickettsiae stain uniformly red. Rickettsiae washed once stain very faintly and those washed more thoroughly do not stain at all with pyronine.

Recently Callot and Vendrely (10) studied the effect of desoxyribonuclease and ribonuclease on rickettsiae. They found that after desoxyribonuclease the staining with Giemsa was greatly reduced, but no marked decrease in staining was detected after digestion with ribonuclease. It is possible that the RNA was washed out during incubation in the control, or that they were dealing with rickettsiae in a physiological state with low RNA content in the cytoplasm.

Demonstration of Nuclear Structures in Rickettsiae

With the phase contrast microscope two or more dark bodies are visible in the rickettsial rods (Fig. 3). These structures are very similar to the chroma-

tinic bodies in bacteria. In order to determine whether they are nuclear structures like those in bacteria it must be shown that they contain DNA.

(a) *Staining with Basic Dyes.*—In bacteria the nuclear structures can be demonstrated with basic dyes after removal of the RNA of the cytoplasm. This is accomplished either with ribonuclease (11) or through hydrolysis with 1 N HCl (12). Robinow (13) hydrolyzes in 1 N HCl and then stains with Giemsa.

Rickettsiae in fresh yolk sac smears stain solidly with basic dyes such as basic fuchsin (Macchiavello's procedure) and pyronine. Rickettsiae which have been washed with saline before fixation lose the ability to stain with these dyes. If washed rickettsiae, or rickettsiae hydrolyzed with 1 N HCl at 60° for 10 minutes are stained with Giemsa chromatinic bodies become apparent (Fig. 4).

Methyl green is a basic dye with high specificity for DNA. Washed rickettsiae were stained with methyl green pyronine. The nuclear structures stained purplish and the cytoplasm faintly pink. Photographed at 630 m μ near the absorption maximum of methyl green, the nuclear structures were clearly visible. The chromatinic bodies, however, appeared most distinct after treatment with ribonuclease and staining with basic dyes. Fig. 2 shows rickettsiae stained with methyl green pyronine after ribonuclease treatment. The nuclear structures stained purplish and stand out clearly in the practically colorless cytoplasm. Fig. 6 is a photograph from the same slide, but taken with the phase contrast microscope.

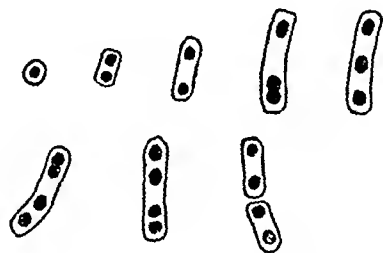
(b) *Ultraviolet Absorption.*—Photographs of washed rickettsiae at 2537 Å show strongly absorbing structures inside the rickettsial bodies (Fig. 5), corresponding to the structures staining with basic dyes. This is further evidence for the presence of nucleic acid in these structures.

(c) *Feulgen Reaction.*—Yolk sac smears were fixed in Carnoy and stained with the Feulgen reaction (modification of Rafalko (14)). The nuclear structures stained very faintly red. With a green filter (Wratten 74) the small dots of the chromatinic bodies could be seen, but nothing else of the rickettsiae was visible. Though the stain was so weak that by itself it would be questionable as a demonstration of DNA, it indicated that the DNA found in purified rickettsiae must be concentrated in these small structures inside the rickettsial bodies. The absolute amount of DNA in one rickettsial organism was obviously extremely small.

The behavior of these chromatinic bodies towards basic dyes, especially after digestion with ribonuclease, the absorption at 2537 Å, and the Feulgen staining therefore leave little doubt that the DNA found in rickettsiae is localized in definite nuclear structures. Spherical rickettsiae contain one nuclear body. In rod-shaped rickettsiae one finds two bodies which are close together in short rods and farther separated in long rods. Sometimes long rods may contain

three or four chromatinic bodies. These are usually spherical, but occasionally one sees dumbbell-shaped structures which suggest a chromatinic body in the process of division (Text-fig. 1, and Figs. 6 and 9).

Electron microscope photographs of rickettsiae washed with saline revealed internal structures which correspond to the chromatinic bodies described above.¹ Rickettsiae with one, two, or three chromatinic bodies were common (Figs. 7 to 12). Sometimes two nuclear structures were very close together, possibly representing the division of a chromatinic body (Figs. 9 and 10). Plotz *et al.* (4) described structures which seem to be identical with our chromatinic bodies.



TEXT-FIG. 1. Nuclear structures in various forms of *Rickettsia prowazekii*. Compare with Figs. 6 and 7 to 12.

SUMMARY

Internal structures of rickettsiae seen with phase contrast microscopy and in the electron microscope contain desoxyribonucleic acid and are therefore nuclear structures similar to those found in bacteria. They are minute spherical bodies, either single as in spherical rickettsiae or varying in number from 2 to 4 in rod-shaped forms. Occasional dumbbell-shaped chromatinic bodies are thought to represent these structures in the process of division. The presence of ribonucleic acid in the cytoplasm of rickettsiae was demonstrated with the use of ribonuclease and basic dyes. Rickettsiae therefore have a cellular organization similar to that of certain bacteria, with a clear differentiation into nuclear structure and cytoplasm.

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¹ The electron microscope photographs were prepared by Dr. E. G. Pickels, formerly at the Rockefeller Institute.

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EXPLANATION OF PLATES

PLATE 35

FIGS. 1 and 2. Rickettsiae in yolk sac smears, stained with methyl green pyronine. Fig. 2 shows the rickettsiae after treatment with ribonuclease, Fig. 1 in the control slide. In the control the cytoplasm is stained intensely with pyronine. After ribonuclease treatment only the nuclear structures are stained. Zeiss 2 mm. NA 1.3 objective, $\times 2400$.

FIG. 3. Photograph of unwashed rickettsiae in yolk sac smear taken with the phase contrast microscope. Spencer 1.8 mm. dark medium, $\times 2700$.

FIG. 4. Rickettsiae in yolk sac smear, hydrolyzed with N HCl 10 minutes, stained with Giemsa. Zeiss 2 mm. NA 1.3 objective, $\times 2400$.

FIG. 5. Rickettsiae washed with saline, photographed in ultraviolet light (2537 Å), Zeiss 1.7 mm. quartz objective, *ca.* $\times 2500$. The nuclear structures absorb more intensely than the cytoplasm.

FIG. 6. Rickettsiae in yolk sac smear, digested with ribonuclease, stained with methyl green pyronine, photographed with the phase contrast microscope. Spencer 1.8 mm. dark medium objective, $\times 2700$. The chromatinic bodies stand out most sharply with this technique.

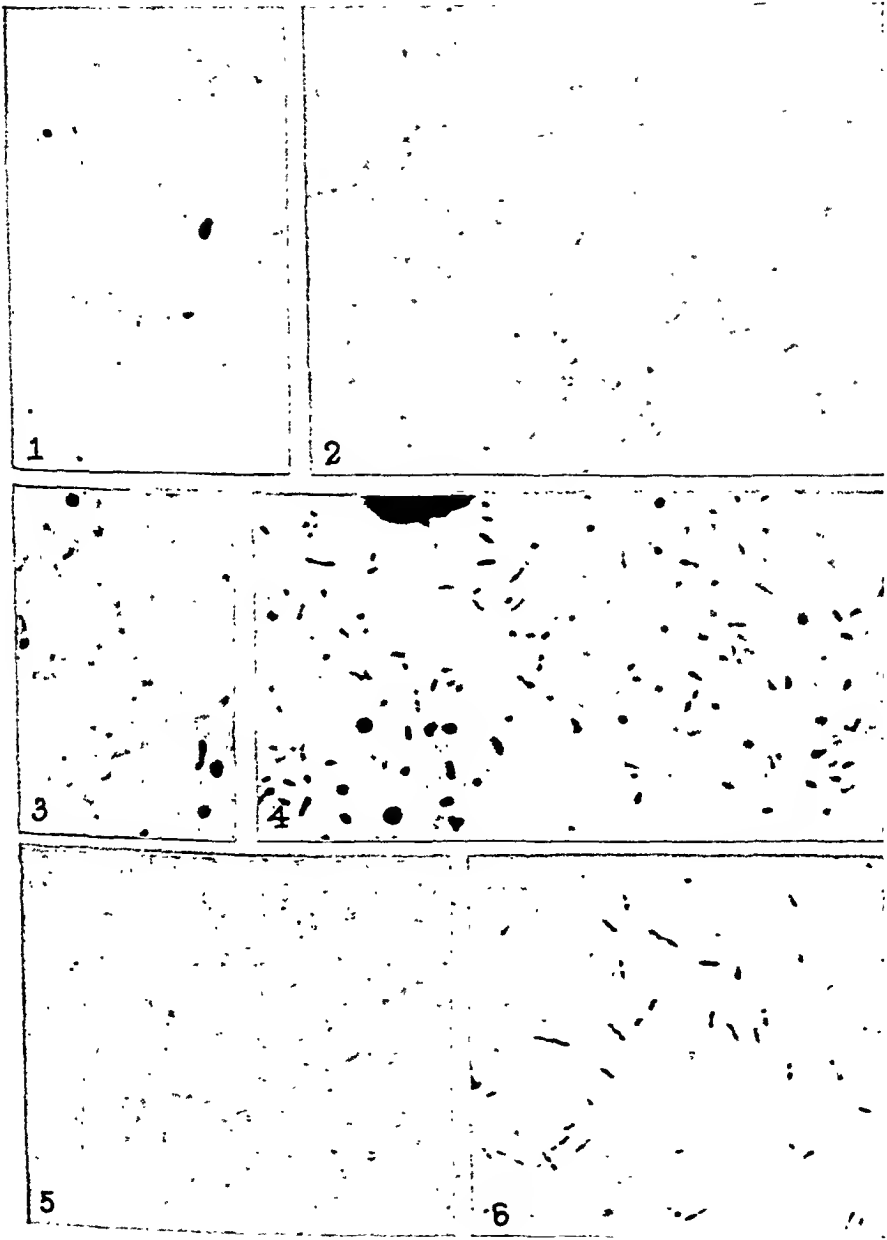
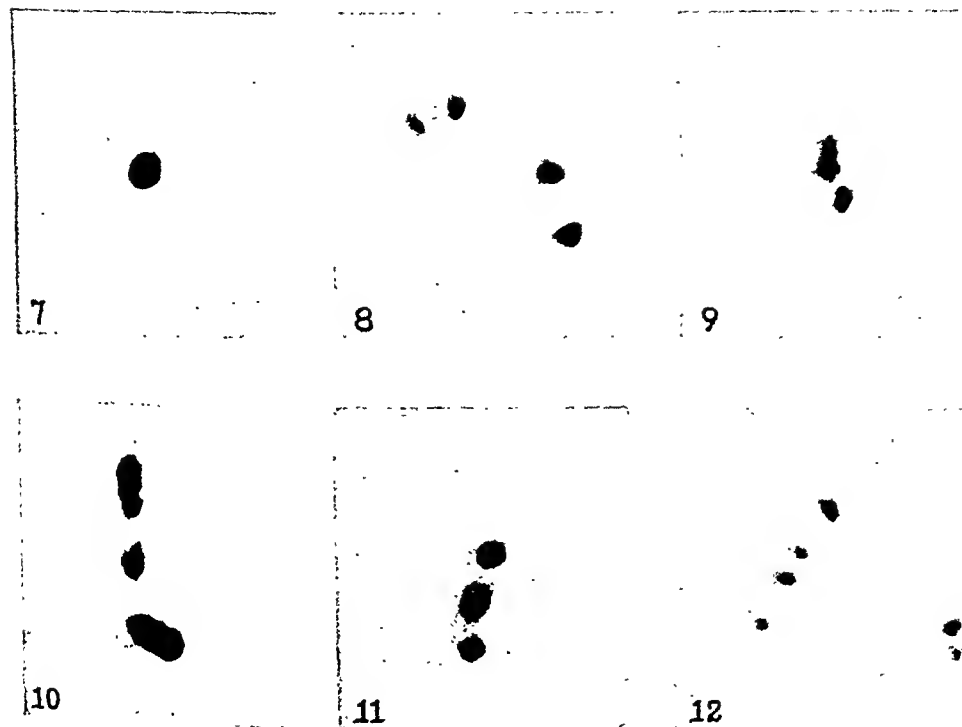


PLATE 36

FIGS. 7 to 12. Electron microscope photographs of rickettsiae extracted from yolk sacs, sulfate-precipitated, and inactivated with merthiolate. RCA Universal electron microscope, *ca.* $\times 15,000$. Compare with Fig. 6.



INDUCTION OF CARDIAC LESIONS, CLOSELY RESEMBLING THOSE OF RHEUMATIC FEVER, IN RABBITS FOLLOWING REPEATED SKIN INFECTIONS WITH GROUP A STREPTOCOCCI

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PLATES 37 TO 42

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Elucidation of the rôle of group A streptococci in the pathogenesis of rheumatic fever might be furthered if a host alteration closely simulating this disease could be induced in laboratory animals infected with these microorganisms; but to date efforts in this direction have failed. This failure possibly stems from one or more factors: (1) lower animals may be incapable of developing the disease; (2) the streptococci employed may have been unable to induce the characteristic host alterations; (3) the experimental conditions may have been unsuitable.

Because a spontaneous disease closely resembling rheumatic fever has not been found in lower animals, its experimental induction might be impossible. The streptococci usually pathogenic for animals belong to serological groups other than A, whereas group A streptococci are chiefly pathogenic for man, and in so far as is known this group comprises the only streptococci that induce the respiratory infections preceding rheumatic fever; the host-parasite relationships among lower animals and streptococci may not be reflected in a rheumatic fever-like state. In experimental streptococcal infections, single strains have usually been employed; but valid data indicate that successive group A streptococcal infections in one person are probably caused by different serological types (1, 2). Rheumatic fever, moreover, occurs among patients in an age period and under conditions which make it probable that they had experienced one or more previous streptococcal infections.

In investigating possible relationships between rheumatic fever and various states of altered reactivity induced experimentally in animals infected with streptococci, workers in this laboratory observed the following phenomena: (a) focal infections of rabbits with viridans, group A or C streptococci resulted in the development of clear cut cutaneous and general hyperreactivity to the homologous infecting strain (3, 4), which was markedly enhanced by frequently repeated minute intracutaneous inoculations (5); (b) intravenous injections of living viridans streptococci (6), or of heat-killed vaccines of group A or C streptococci (5) induced in rabbits a state of diminished cutaneous reactivity

* This work was done during the tenure of a Life Insurance Medical Research Fellowship.

to inoculation with homologous strains; (c) these immunized animals simultaneously showed cutaneous hyperreactivity to strains belonging to heterologous groups (4, 7); (d) some rabbits immunized intravenously or subcutaneously with heat-killed vaccines of one type of group A streptococci developed decreased skin reactivity to intracutaneous inocula of the same type, but simultaneously showed greater than normal cutaneous reactivity to minute intracutaneous inocula with heterologous types, and the same often held true when the preliminary immunization was induced by repeated skin infections with a strain of a very rabbit-virulent group A streptococcus (8), and the state of cutaneous hyperreactivity was brought out far more clearly with high dilutions of the challenging inocula than with low dilutions; (e) rabbits resting 2 or more months from immunization with viridans, group A or C streptococci became cutaneously and systemically hyperreactive to the homologous strain of streptococci previously injected (7). The following information was also available: Rheumatic fever patients develop type-specific antibodies to the group A streptococcus inducing the nasopharyngeal infection preceding a rheumatic fever attack (1, 2); rheumatic fever patients, as a rule, were found to be hyperreactive to viridans and group A streptococcal nucleoproteins (9) and to group A streptococcal vaccines (10). It was later assumed that this hyperreactive state was induced by recurring focal (nasopharyngeal) infections with a succession of different serological types of group A streptococci.

These observations suggested that a rheumatic fever-like state might be induced in animals by successive focal group A streptococcal infections, each caused by a serological type heterologous to those previously employed. This communication reports the first testing of this hypothesis.

Methods

Because it was desirable to test a relatively large group of animals, and as considerable information was available concerning the reactivity of rabbits to streptococcal infections, this species was chosen. New Zealand Reds and a cross-breed designated hare brown, all bred in the Rockefeller Institute, were usually employed; occasionally chinchilla and other varieties were tested. All rabbits were fed approximately 560 gm. of Rockland rabbit ration pellets within each week. Animals with skins which mostly remained bare for considerable periods after close clipping were preferred; and such clipped sites were used primarily; but after repeated inoculations it sometimes became difficult to find very suitable skin, and coarsely hairy areas had to be inoculated.

The group A streptococci employed all exhibited matt or mucoid colony forms in 18 to 24 hours growth on moist rabbit blood agar; and were shown to produce large amounts of type-specific M protein in Todd-Hewitt broth made with neopeptone. Mostly they had only moderate virulence for rabbits, but even in this respect there was considerable variation, both among the various types employed and in different subcultures of single strains. Efforts to increase virulence by rabbit passage have been only moderately successful.

Sixteen to 20 hour Todd-Hewitt neopeptone broth cultures were serially diluted in tenfold steps with broth, and the inocula in 0.1 cc. volume, containing between 10^{-8} and 10^{-2} cc. of the original culture, were injected into closely clipped skin of right and left gluteal, lumbar, thoracic, or shoulder areas. Ten times more cocci were injected on the right side than on the

eft. In the original groups of animals, the 2nd to 4th successive focal cutaneous infections are set up in the same well healed, but scarred, gluteal sites. Subsequently, because of re-

TABLE I
Rabbit 70-58—New Zealand ♀

Infections					Course of infections				
Date	Strepto- coccus type	Inocula	Skin site	Skin reaction	Date	Weight	ESR	ASO units	Remarks
1945					1945	kg.	mm./ hr.		
7/17	1	10 ⁻³	Gl	N	7/17	3.0			
8/28	11	10 ⁻⁵	Gl	Initially >N; <N in few days	8/19	3.1			
10/ 8	17	10 ⁻⁵	Gl		10/30		1		
11/12	13	10 ⁻⁴	Gl		11/12	3.8		<25	
11/15	13	10 ⁻⁴	Gl		11/16		1		
11/19	13	10 ⁻³	Gl		11/25		1	25	
11/25	13	10 ⁻³	Gl		11/28		1	25	
1947					1947				
1/13	3	10 ⁻⁴ , 10 ⁻⁵	Gl; Th	>N	1/10	3.7			
					1/18		1	25	
					1/20	3.8			
4/18	19	10 ⁻⁴ , 10 ⁻⁵	Gl; Sh	>N	4/16	4.0			
					4/25		1	50	
5/28	17	10 ⁻³ , 10 ⁻⁵	Gl; Lu	<N	5/27			25	
9/16	1	10 ⁻⁴ , 10 ⁻⁵	Gl; Th	>>N	6/ 7		1	25	
					9/19				*
					9/22				Anorexia
					9/25		130	400	"
					9/26		47	>1000	"
					9/27		76		" +
					9/29				Died‡

N, average cutaneous response of normal control rabbits to intracutaneous inoculation with streptococci.

Gl indicates gluteal; Lu, lumbar; Th, thoracic; Sh, shoulder.

* Erythema over right knee.

ESR, erythrocyte sedimentation rate (Westergren).

† Negative blood culture.

ASO, antistreptolysin O titer.

‡ Autopsy blood culture negative.

E.S.R. of normal rabbits, 1 to 2 mm. per 1 hour and 2 to 4 mm. per 2 hours.

sults recorded below, each reinfection, usually with a type of streptococcus not previously injected, included 4 areas: right and left scarred gluteal skin sites and a right and left skin site least likely to have been locally inflamed in previous infections.

All skin lesions were measured daily until recession was demonstrated. The condition of the rabbits was observed; and their weights were recorded at suitable intervals. Blood was obtained from the ear veins for serum which was refrigerated and later tested for antistreptolysin O and antistreptokinase content, and for precipitin reactions with extracts of homologous and heterologous types of group A streptococci. These data furnished a rough index of the serological responses to the infections. During the earlier experiments erythrocyte sedimentation rate determinations (Westergren method) were made once or twice during the

TABLE II
Rabbit 71-77—Hare Brown ♂

Infections					Course of infections					
Date	Streptococcus type	Inocula	Skin site	Skin reaction	Date	Weight	ESR	WBC 1000	ASO units	Remarks
1947					1947	kg.	mm./hr.			
5/8	19	10^{-4} , 10^{-4}	Gl;	Sh—>2 other norms	5/8	2.7				
6/20	1	10^{-4} , 10^{-5}	Gl;	Lu—<<N	6/20				<25	
9/16	1	10^{-4} , 10^{-5}	Gl;	Th—<<N						
11/6	London	10^{-3} , 10^{-4}	Gl;	Th—=N	11/4				<25	
					11/19		3		75	
1948					1948					
2/6	3	10^{-3} , 10^{-4}	Gl;	Th—<N	1/16		1	7.7	25	
					2/6	3.4				
					2/14	2.8	152	10.8	600	Anorexia, marked, cardiac irregularity* died*

* Negative blood culture ante- and postmortem.

fortnight following the inoculations; but later both erythrocyte sedimentation rate determinations and leucocyte counts were made twice or three weekly until they were approximately normal or until the animal died or was sacrificed.

Where indicated, blood cultures were made from living rabbits with blood obtained from ear veins and placed both in Todd-Hewitt neopeptone blood broth and on rabbit blood agar plates. Blood obtained postmortem from the inferior vena cava of all dead rabbits was similarly cultured; and streptococci recovered were identified serologically.

During the first 4 to 5 months' experimentation it was found that successive monthly to bimonthly inoculations with streptococci of different serological types into the same gluteal skin sites usually induced progressively smaller local lesions than those which followed similar inoculation in comparable skin of normal controls; but in their previously uninfected (e.g.

thoracic) skin the same sized inoculum almost invariably induced greater local inflammation than in their multiply infected gluteal skin or in the thoracic skin of normal controls (Table I;

TABLE III
Rabbit 71-80—Hare Brown ♀

Infections					Course of infections						
Date	Streptococcus type	Inocula	Skin site	Skin reaction	Date	Weight	ESR		WBC 1000	ASO units	Remarks
							1 hr.	2 hr.			
1947					1947	kg.	mm.				
5/28	6	10^{-3} , 10^{-4}	Gl; Lu	>2 other norms	5/28	3.4					
					6/ 7	2.7				700	
6/20	1	10^{-4} , 10^{-5}	Gl; Th	<N	6/20	2.8				<25	
9/16	1	10^{-4} , 10^{-5}	Gl; Th	<N	9/30		1			100	
11/ 6	London	10^{-3} , 10^{-4}	Gl; Sh	=N	11/ 3					100	
					11/14	3.3					*
					11/18	3.2	1			75	†
1948					1948						
2/ 6	3	10^{-3} , 10^{-4}	Gl; Th	<N	1/19	3.4	1		16.2	25	
					2/16	3.4	4		19.3	50	
					2/19		2		14.2	75	
					2/25		3		13.5	25	
9/21	9	10^{-3} , 10^{-4}	Gl; Th	=N	5/26	3.5					
9/22	9	10^{-3} , 10^{-3}	Lu		9/21	3.5	1		18.0		
					9/22				22.5		
					9/23		5	11		<25	
					9/24	3.1	7	16	18.9	<25	
					9/26	2.9					
					9/27	2.7	29	56	30.1	50	
					9/28	2.7	33	54	21.8	50	Anorexia
					9/29		49	80		75	Diarrhea
					10/ 1	2.3	45	88	19.5	200	
					10/ 2	2.4				100	Sacrificed§

* Guards hind legs.

† Negative blood culture.

§ Autopsy blood culture negative.

January, 1947). It was, therefore, obvious that new areas were requisite to obtain a rough approximation of an animal's cutaneous reactivity to successive inoculations. Furthermore, because of this finding that succeeding streptococcal skin inflammation was likely to be more

intense in previously uninflamed skin than in scarred sites, it seemed possible that streptococci might survive longer and effect more sustained local infection in fresh skin than in scarred areas; and that from the larger inflammatory zones more toxic material might be elaborated and absorbed than from the small lesions.

Variations in the inoculation procedures are illustrated in Tables I, II, and III. Occasionally, as in November, 1946 (Table I), or in September, 1948 (Table III), repeated intracutaneous inoculations with the same type of streptococcus were given within a few days in attempts to enhance the infectious stimulus of lowly virulent strains. In other instances, as with type 1 (Tables I, II, and III), the same type was reinjected after a relatively long interval; but generally each successive inoculation in a given animal was with a type heterologous to those previously used to infect a given animal.

Autopsies were performed as soon after death as possible; those on sacrificed rabbits were carried out immediately after exitus, usually effected with intravenous sodium nembutal. Tissues were fixed in Zenker-acetic acid, and sections cut from paraffin blocks were stained with hematoxylin and eosin, Giemsa, Weigert-hematoxylin and eosin, Masson's trichrome, Mallory's aniline blue, and where indicated with Gram-Weigert and malachite green-acridine red (11), a technique applicable to Zenker-fixed tissues, whereas the Unna-Pappenheim methyl green-pyronine technique requires alcohol fixation.

RESULTS

After sustaining 2 to 10 infections with streptococci of different serological types within 3 to 20 months, some rabbits sickened and showed various combinations of the following signs and symptoms: elevated erythrocyte sedimentation rates for 1 to 2 weeks; leucocytosis; anorexia; weight loss; postexertional dyspnea; occasional transient pulmonary rales; tachycardia; and in a few instances, definitely irregular cardiac rhythm. Many of these rabbits recovered; a portion were sacrificed within 10 to 14 days following their last infection while exhibiting definite symptoms, leucocytosis, and elevated erythrocyte sedimentation rates higher than were occurring in normal controls; in several rabbits, however, a severe illness developed following the last streptococcal infection and terminated fatally, whereas some of the normal controls survived the same streptococcal infection. A few in the fatal group died within 2 to 5 days following the last infection (even though normal controls in some instances survived) and in all except one of these rapidly fatal cases, streptococcal bacteremia was established at autopsy. In about half of a group of rabbits dying spontaneously between 6 and 14 days after the last infection, streptococcal bacteremia was demonstrated at autopsy; in the other rabbits of this group, however, streptococci could not be cultured from the blood either before death or at autopsy.

In the hearts of the successively infected rabbits which had sickened and succumbed, and of those sacrificed while sick, there have been found on microscopic examination focal alterations in the connective tissue framework in blood vessel adventitia, valves, mural endocardium, epicardium, and in the myocardial interstitium. Many collagen fibers in these sites are swollen; some are intensely eosinophilic, others stain poorly; some swollen collagen fibers stain entirely, whereas others stain in patchy fashion like fibrin with both Masson's trichrome and Mallory's connective tissue techniques. Arranged about and interspersed

in fields of swollen "fibrinoid" collagen are nodular collections of large, irregularly shaped cells, often with abundant, finely granular basophilic cytoplasm which takes a smudgy red color with the malachite green-acridine red stain. Often these cells have very indistinct outlines; some have long streamer-like cytoplasmic processes which gradually fade into the contiguous areas. The vesicular nuclei, single or multiple, are variously shaped, and have sharply defined membranes. Clumping of chromatin often leaves the rest of the nucleus clear. Some nuclei are pyknotic. Cells with multiple, centrally placed nuclei, 2 to 10 in number, occur in greatest profusion in the mitral and aortic sulci and rings and in the endocardium (Fig. 3). The lesions also contain many cells of the Anitschkow myocyte type, and occasionally small round cells and polymorphonuclear leucocytes, both pseudo- and true eosinophiles. The sites of predilection for the occurrence of these nodular granulomata in most hearts are endocardial, subendocardial, and blood vessel adventitia and paraadventitia. These adventitial lesions are by no means limited to the roots of the valves, but at times are conspicuously present throughout the hearts, particularly in the left ventricle and interventricular septum.

In some hearts the granulomata occur in the loose myocardial interstitium unassociated with arteries or veins but, in most instances, with capillaries. In agreement with Gross (12) the latter are designated "myocardial granulomata" to distinguish them from granulomata associated with other cardiac structures. Interstitial valvulitis, most marked in the middle of the cusps, has occurred commonly in the mitral and aortic valves and also in the right auriculoventricular valve; these areas beneath the line of closure show edema of varying intensity and cellular components like those in the submiliary nodules. Marked proliferation of mitral and aortic valvular endocardial and subendocardial cells occurs in several hearts to create many layered palisades containing numerous multinucleated giant cells dispersed in swollen or "fibrinoid" collagen (Figs. 1 and 2). These lesions are occasionally limited to the sulci, but are also found often on both surfaces of the valve and of the chordae tendineae (Fig. 10). At times the most superficially palisaded cells have apparently undergone necrosis and conversion into acellular material that stains like fibrin. The latter phenomenon was most marked in rabbits dying spontaneously within 2 weeks after the final infection. On no valves were there seen, macroscopically, rows of fine verrucae along the lines of closure. In the gross, however, the mitral valve of several rabbits showed along the line of closure a row of fine discrete opalescent elevations usually more marked on the aortic leaflet. Microscopically these elevations consist of interstitial edema and valvulitis which in some instances are more intense than in the neighboring tissues. Occasionally larger fine, firm white nodules projecting from the surface of the valve were visible. Foci of frankly "fibrinoid" collagen¹ are seen in auricular (Fig. 12) and ventricu-

¹ The expression "frankly fibrinoid collagen" indicates that the altered collagen stains unequivocally like fibrin with Mallory's connective tissue and Masson's trichrome techniques.

lar epicardium in association with proliferated epicardial and subepicardial elements. These patches of epicarditis are microscopic in size; and no extensive plastic pericardial exudate has been detected in the gross.

Granulomata in the compact paravascular connective tissue differ in architectural configuration from the "myocardial granulomata" in the looser tissue between muscle bundles. There are submiliary granulomata closely resembling the coronal (Fig. 6), reticular (Figs. 4 and 7), and mosaic (Figs. 8, 9, and 11) types of Aschoff bodies described by Gross in human rheumatic hearts (12); and in the left ventricle and interventricular septum of a few rabbit hearts several myocardial granulomata are often seen in a low power field; but in no rabbit dying spontaneously or sacrificed within 2 weeks after final infection, have there been found well developed polarized or fibrillar types of granulomata. Gross considered the peculiarly shaped and arranged cells in such Aschoff bodies to represent terminal metamorphosis of the rheumatic granuloma cells into fibroblasts. Damage to myocardium adjacent to granulomata has been prominent, and has ranged from swelling and vacuolation of the myofibers and vesiculation of their nuclei to complete dissolution and replacement by scar. Occasionally apparent fusion of neighboring granulomata combined with extensive adjacent myocardial destruction and connective tissue replacement has resulted in macroscopically visible lesions in stained sections of left ventricle and papillary muscles.²

The coronary arterial system is variously altered. Fairly commonly there is marked intimal hyperplasia and elastification involving chiefly small arteries and arterioles. A well developed intimal musculoelastic hyperplastic lesion occurs in several rabbits. In the hearts of two rabbits there is found marked ramification of fibrinoid material throughout or surrounding the wall of a small artery or capillary (Fig. 5). Interspersed in and arranged about the extension of this intensely eosinophilic material into the tissue adjacent to the vessel are granuloma cells of the type found in the previously described rabbit granulomata. This vascular lesion closely resembles that described in rheumatic human hearts by Pappenheimer and Von Glahn (13). Panarteritis of the so called "allergic" or periarteritis nodosa type is conspicuously absent in the hearts of all intracutaneously infected rabbits. Verrucous and polypoid endarteritis are occasionally present. In the intima and immediately subadjacent media of the aorta near its root there occasionally is seen a lesion comparable with that in the valve sulci and closely resembling that described by Pappen-

² A striking increase in size of the adrenal glands occurred in rabbits dying, or sacrificed while sick, following the last of several intracutaneous streptococcal infections. Microscopically hyperplasia, hypertrophy, and necrosis of fascicular zone cells are seen. There is striking correlation between the degree of macroscopic enlargement of the fascicular zone of the adrenal cortex and the occurrence of myocardial granulomata. Detailed data concerning these observations will be presented shortly.

heimer and Von Glahn (13) in human rheumatic aortitis. Occasionally there are foci of clearly defined fibrinoid collagen in the adventitia of the root and first portion of the aorta. These lesions will be illustrated later.

Neither bacteria nor any structures resembling inclusion bodies have been seen in the above described lesions stained according to Gram-Weigert or Giemsa techniques. There has, moreover, been no calcification of the myocar-

TABLE IV

Rabbit groups	No.	Bacteremia	Acute rheumatic fever-like cardiac lesions	Myocardial scars or healed arteritis of rheumatic type
1. Normal rabbits.....	8	—	0	0
2. I.v. vaccine (group A or C streptococci)....	8	—	0	0
3. Dying 1-18 days after 1 i.v. infection.....	20	20	0*	0
4. Sacrificed 1 and 4 mos. after 1 i.v. infection..	2	0	0	0
5. Sacrificed within 1 mo. after 1 i.c. infection..	4	0	0	0
6. Dying within 2 wks. after 1 i.c. infection....	13	12	0	0
7. Dying 3 wks. after 1 i.c. infection.....	1	1	1†	0
8. Sacrificed 10 to 21 days after last of several i.c. infections..	37	0	7	8
9. Dying 2 to 5 days after 2nd i.c. infection....	3	2	3‡	0
10. Dying 8 to 14 days after last of 5 to 9 i.c. infections.....	3	0	3	2
11. Dying 5 to 9 days after last of 2 to 9 i.c. infections.....	7	7	6	3
12. Dying several weeks after last of several i.c. infections.....	4	0	0	3
Totals.....	110	42	20	16

i.v., intravenous. i.c., intracutaneous.

* The hearts of two rabbits dying 12 and 18 days, respectively, after one i.v. inoculation show an acute exudative and necrotizing arteritis.

† Interstitial valvulitis marked; vascular adventitial and interstitial foci of lymphocytes and plasma cells and occasional foci of young mesenchymal cells in adventitia without demonstrable alteration of collagen; no necrotizing arteritis.

‡ Slight interstitial valvulitis only.

dial lesions, a very conspicuous phenomenon in experimental myocarditis induced with filterable viruses.

Controls.—The tissues of rabbits of the same stock and breeds, both normal and subjected to various experimental procedures have been examined at intervals during the investigative period in order to learn whether comparable lesions were occurring in such control animals, for it should be recalled that a peculiar myocarditis was described by Loewe and his coworkers (14) among stocks of rabbits injected with various materials as well as among uninoculated

controls. These workers ascribed these lesions to a spontaneous epidemic in their stock. As indicated in Table IV, enough controls have been examined to eliminate fairly certainly the possibility that such an epidemic existed among the animals we used. The small focal lesions described by Miller (15) in rabbits' hearts have not been encountered among our present stock of experimental animals or controls; hence it seems probable that the cardiac lesions that developed in our animals bear no relationship to previously described spontaneous rabbit myocarditis.

Inspection of Table IV indicates that the cardiac lesions forming the basis for this report have developed only in rabbits that had undergone multiple, successive cutaneous infections with group A streptococci of different types. In most of the animals showing these lesions, ante- and postmortem blood cultures were negative, and with bacterial stains no bacterial cells could be seen in the lesions; hence it seems improbable that the fresh tissue alterations were due to a direct action of streptococcal cells at the site of injury. In some of the animals, there was evidence of terminal streptococcemia, but even so those animals dying acutely with streptococcemia following their first cutaneous infection, or from intravenous inoculation with streptococci have not developed these submiliary granulomata. Similar negative results were found in rabbits repeatedly immunized intravenously with heat-killed group A or C streptococcal vaccines, as well as in those sacrificed after one intracutaneous inoculation. It seems, therefore, that those finally dying with bacteremia following the last of multiple skin infections developed these cardiac lesions (in which repeated bacterial stains have been negative) because the final insult affected tissues peculiarly conditioned by previous focal infections. It seems quite possible that in this group of rabbits, the bacteremia was, in fact, a terminal event.

DISCUSSION

The cardiac granulomata described, which in many respects bear such a striking histopathological similarity to those of human rheumatic fever, have been encountered only in animals that had undergone multiple, successive cutaneous infections with group A streptococci of several different types. It, therefore, seems probable that the relatively long experimental period and the reconditioning that the animals' tissues underwent as a result of several focal infections with different types of group A streptococci were important factors in the pathogenesis of these lesions. In certain respects this experimental procedure follows the pattern encountered in rheumatic fever patients: they have successive infections with different types of group A streptococci, and these infections are usually focalized in the upper respiratory tract and accessory tissues. Because it was impractical to infect rabbits' throats and sinuses repeatedly, and because successive focal infections appeared hypothetically to be important, the rabbits' skin was selected for the repeated insults.

The carditis developed, moreover, following infections with the same microorganisms that have been repeatedly proven to occur in the infections that precede attacks of rheumatic fever in man. This unique sequential relationship could not be demonstrated until Lancefield's system of classification of streptococci was available (16). In rabbits made hyperreactive to viridans, group A and group C streptococci by repeated focal infections and then shocked with intravenous inoculations of homologous streptococci, cardiac lesions of this type were not encountered (17).

It seems expedient to compare the carditis herein described in rabbits with that in animals of the same species with serum disease or subjected to repeated parenteral injections of foreign protein. This will be the subject of a later communication; but available evidence seems to indicate that the over-all histopathological picture in the rabbits repeatedly infected with streptococci bears closer resemblance to that of human rheumatic carditis than does experimental serum disease carditis. The fatal termination within 6 to 14 days, of an illness developing after the last of several focal infections is a phenomenon which, to our knowledge, has not been recorded in rabbits repeatedly injected and shocked with foreign protein.

Among the random samples of rabbits subjected to the described experimental procedure, only a small portion have developed these cardiac lesions. It seems pertinent to mention that only a small proportion of human beings in this geographical area develop rheumatic heart disease, and today an even smaller proportion develop polyarthritis rheumatica. Among subjects who have recovered from previous attacks of rheumatic fever and in rheumatic families, the incidence is considerably higher. There has been no attempt to select specially susceptible stock among the animals used in these experiments.

On the basis of evidence derived from the experiments here reported and from studies of rheumatic fever in man, it seems justified to assume that similar host-streptococcus relationships may be operative and requisite in the pathogenesis of these cardiac lesions in rabbits and rheumatic carditis in man.

SUMMARY

Cardiac lesions, closely resembling those found in rheumatic fever, have developed in rabbits that sickened following multiple, successive skin infections with several serological types of group A streptococci.

It is a pleasure to acknowledge the valuable technical assistance of Miss Jeanne Epstein and Mr. Andrew Littell.

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EXPLANATION OF PLATES

The photographs were made by Mr. Julian Carlile and Mr. Richard Carter.

PLATE 37

FIG. 1. Rabbit 73-13, sacrificed 15 days after last of 4 infections; no bacteremia at autopsy. *A*, polypoid endo- and subendocardial proliferation (palisade) in mitral sulcus; *B*, external elastic lamella; *C*, focus of frankly fibrinoid collagen. Weigert-hematoxylin and eosin. $\times 195$.

FIG. 2. Rabbit 71-77 (see Table II),—died 8 days after last of 5 infections; no bacteremia ante- or postmortem. *A*, extensive endo- and subendocardial proliferation (palisade) in aortic pocket; *B*, inflammation in annulus; *C*, aortic interstitial valvulitis; *D*, root of aorta. Weigert-hematoxylin and eosin. $\times 116$.

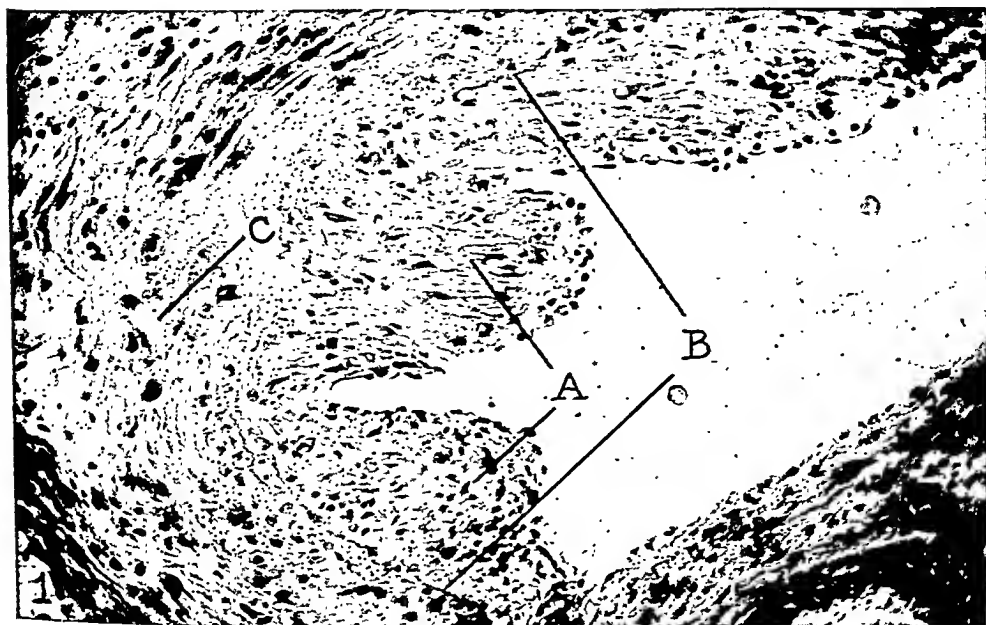
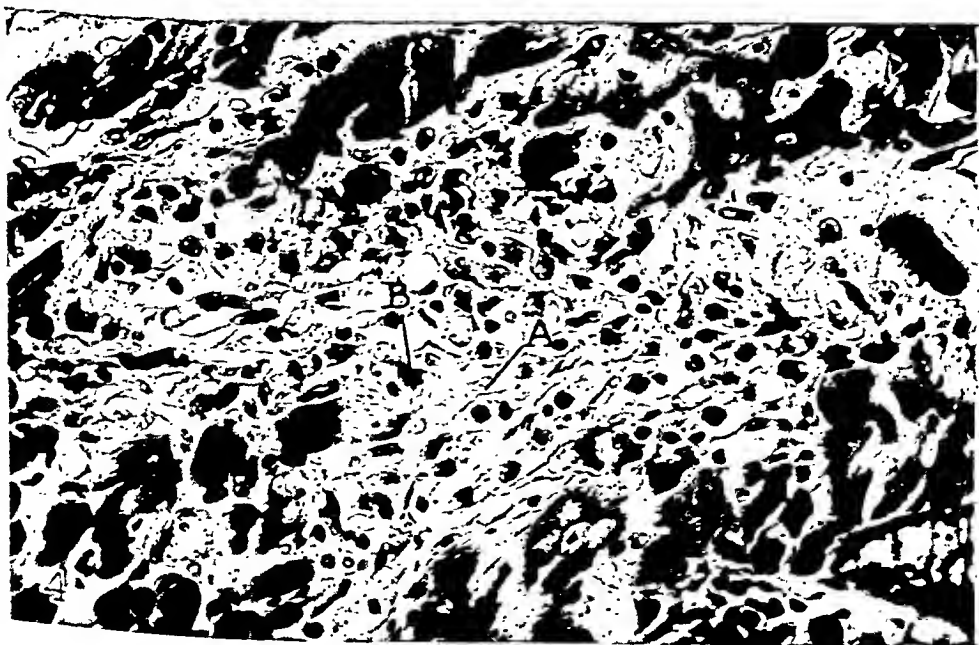
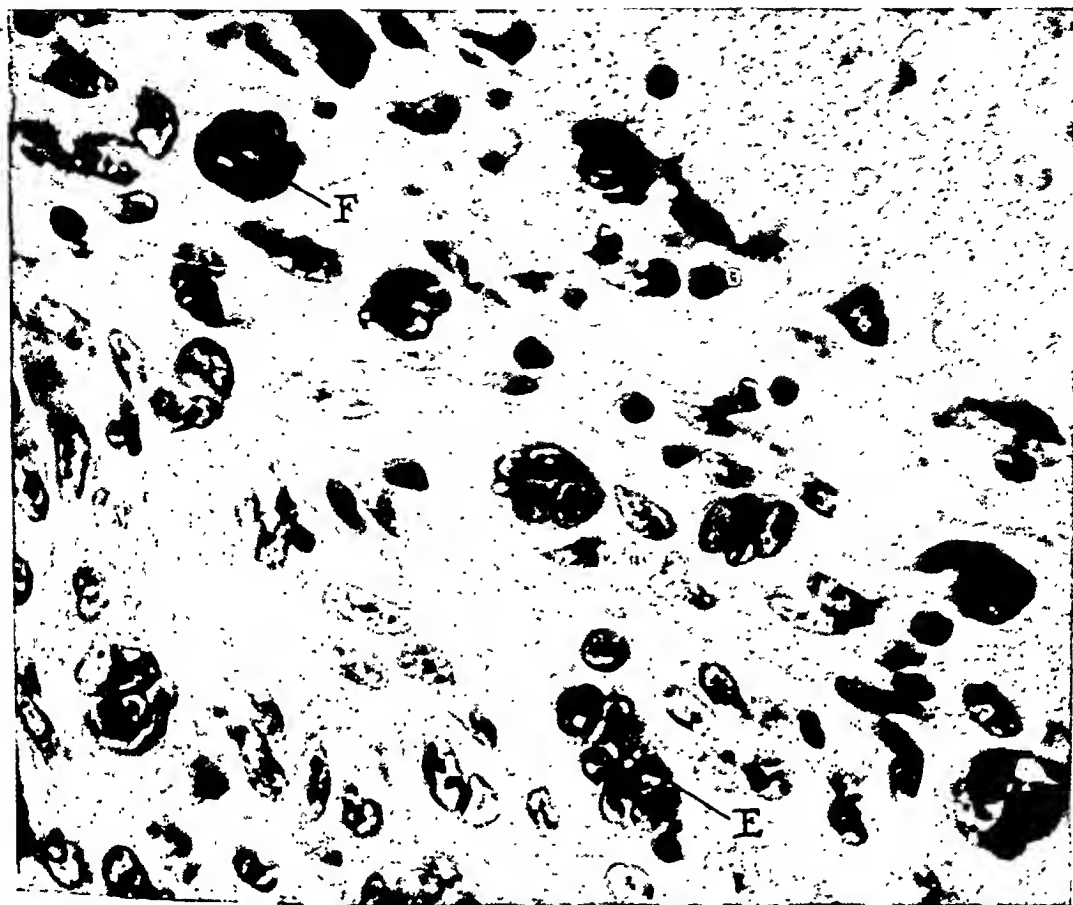


PLATE 38

FIG. 3. Rabbit 71-77,—higher magnification of *A*, Fig. 2; numerous mono- and multinucleated cells, some with bizarre shaped nuclei, basophilic cytoplasm, and indistinct cytoplasmic outline; *E* and *F*, cells with 8 nuclei. Hematoxylin and eosin. $\times 886$.

FIG. 4. Rabbit 71-77,—reticular myocardial granuloma, interventricular septum. *A*, swollen collagen fibers forming interlacing network; collagen framework which assumes a direction roughly parallel with the myocardial bundles; *B*, cell with abundant cytoplasm; necrosis of adjacent myofibers. Weigert-hematoxylin and eosin. $\times 395$.

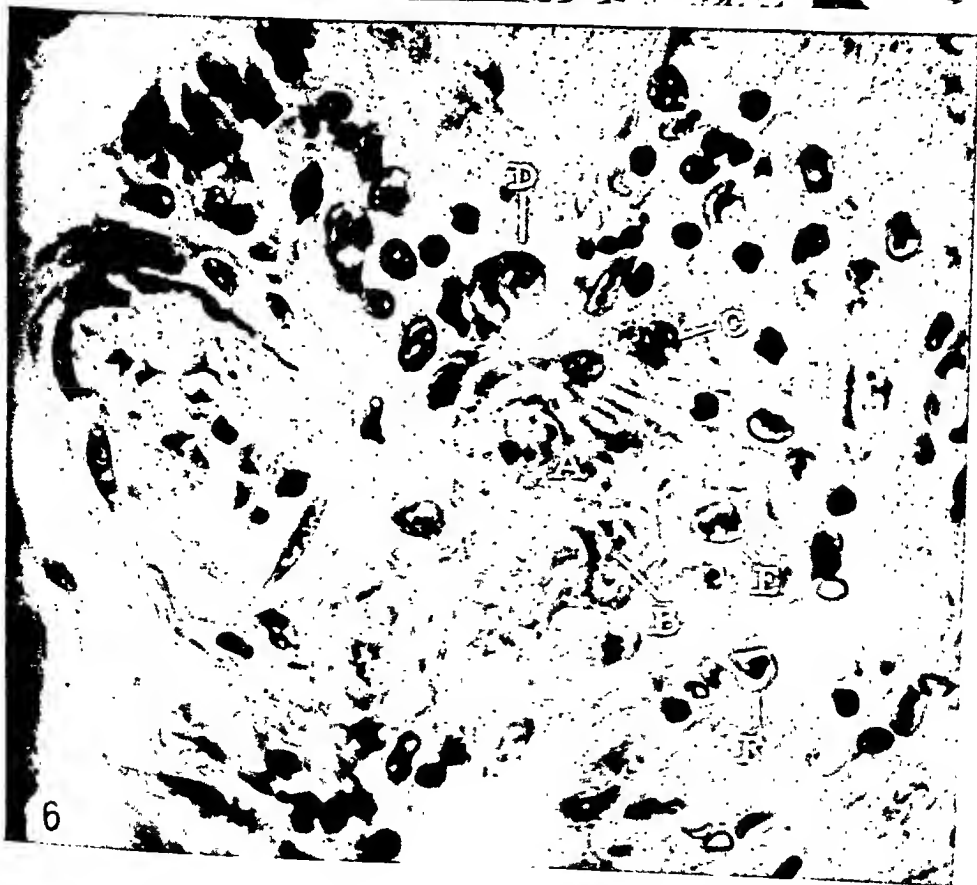
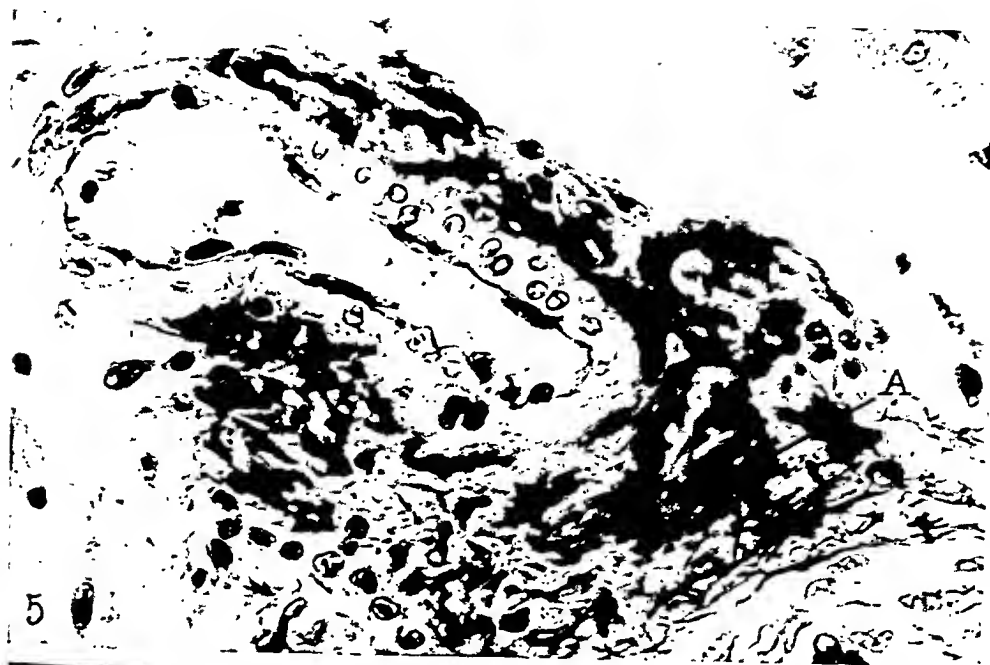


Murphy and Swift. Induction of cardiac lesions.

PLATE 39

FIG. 5. Rabbit 71-77,—artery in left ventricle. *A*, frankly fibrinoid collagen, bordered by granuloma cells, in adventitia and paraadventitia; panarteritis nodosa absent. Weigert-hematoxylin and eosin. $\times 743$.

FIG. 6. Rabbit 71-77,—adventitial and paraadventitial coronal granuloma in inter-ventricular septum. *A*, center of focus of frankly fibrinoid collagen; *B* and *C*, indistinct cell masses; *D*, cell with 3 nuclei; *E*, cell with fibrocytoid nucleus; *F*, cell with owl-eyed nucleus; many cells have indistinct cytoplasmic outlines; panarteritis nodosa absent. Hematoxylin and eosin. $\times 861$.

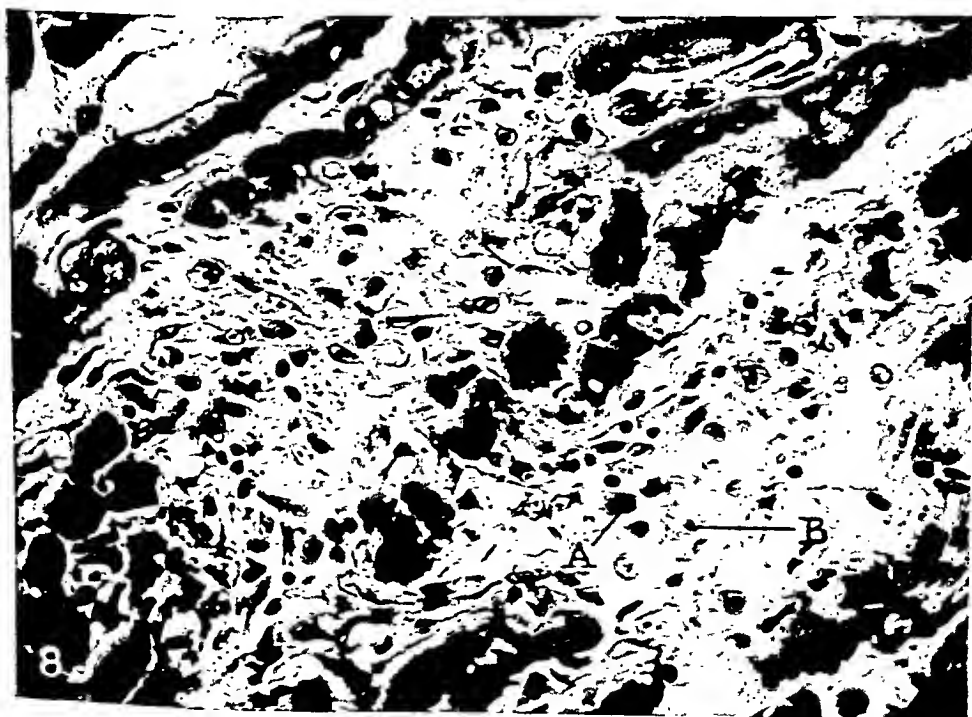
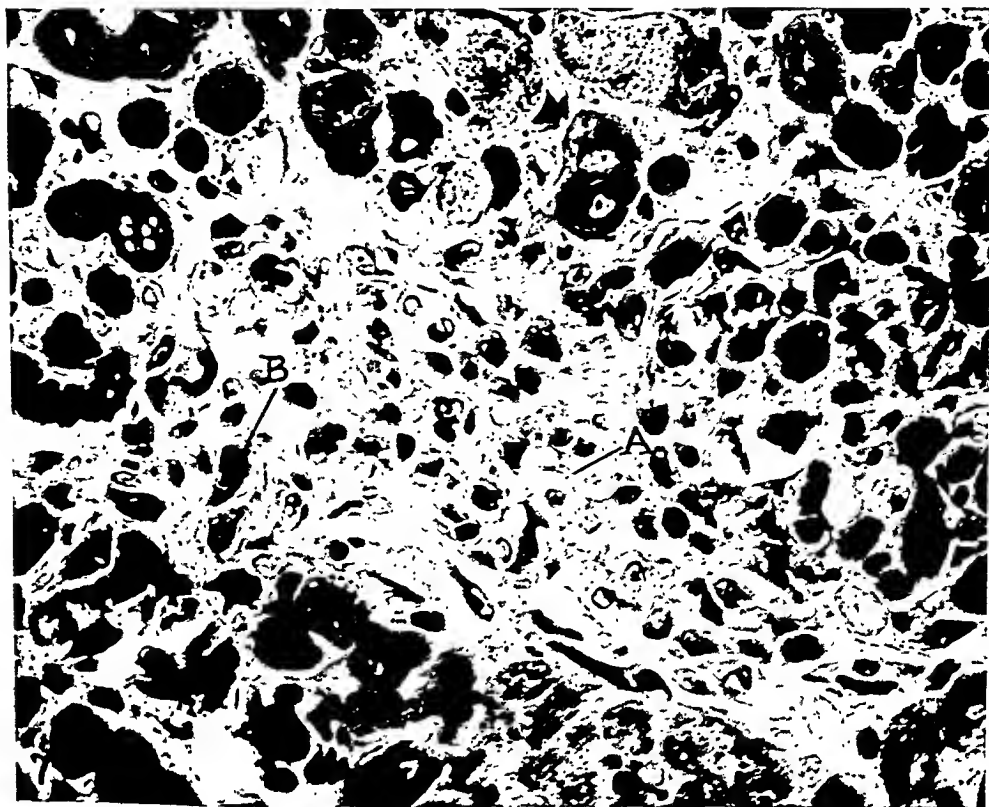


(Murphy and Swift: Induction of cardiac lesions)

PLATE 40

FIG. 7. Rabbit 71-80 (see Table III),—sacrificed 10 days after last of 6 infections; autopsy blood cultures negative; reticular myocardial granuloma in interventricular septum. Cells interspersed in interlacing network of (A) swollen collagen fibers; B, cell with abundant basophilic cytoplasm; vacuolation of nuclei and cytoplasm of adjacent myofibers. Giemsa stain. $\times 621$.

FIG. 8. Rabbit 71-80,—two mosaic myocardial granulomata in left ventricle; granuloma cells lodged between collagen masses. A, cell with 3 nuclei; B, cell with pyknotic nucleus and abundant raggedly outlined cytoplasm; most cells have indistinct cytoplasmic outlines; disintegration of adjacent myofibers. Hematoxylin and eosin. $\times 404$.

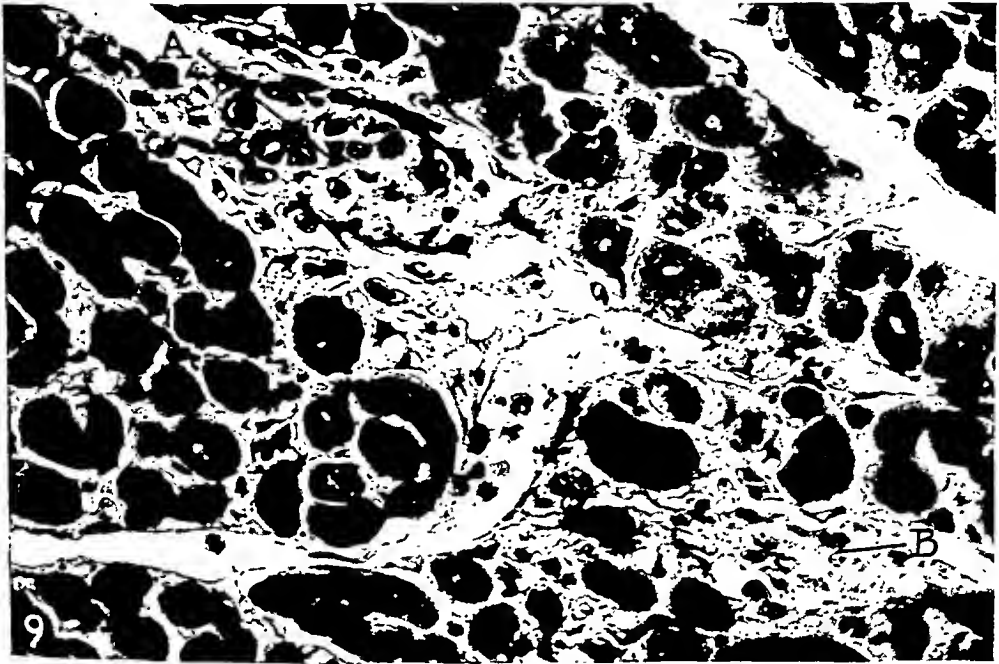


(Murphy and Swift Induction of cardiac lesions)

PLATE 41

FIG. 9. Rabbit 71-80 (see Table III),—left ventricle; *A* and *B*, 2 mosaic myocardial granulomata; foci of frankly fibrinoid collagen in granuloma *A*. Hematoxylin and eosin. $\times 465$.

FIG. 10. Rabbit 71-77,—endocardial nodule on chorda tendineae at mitral leaflet attachment. Numerous multinucleated cells surrounding *A*; many cells with basophilic cytoplasmic streamers surrounding *B*; interstitial inflammation, *C*. Giemsa. $\times 255$.

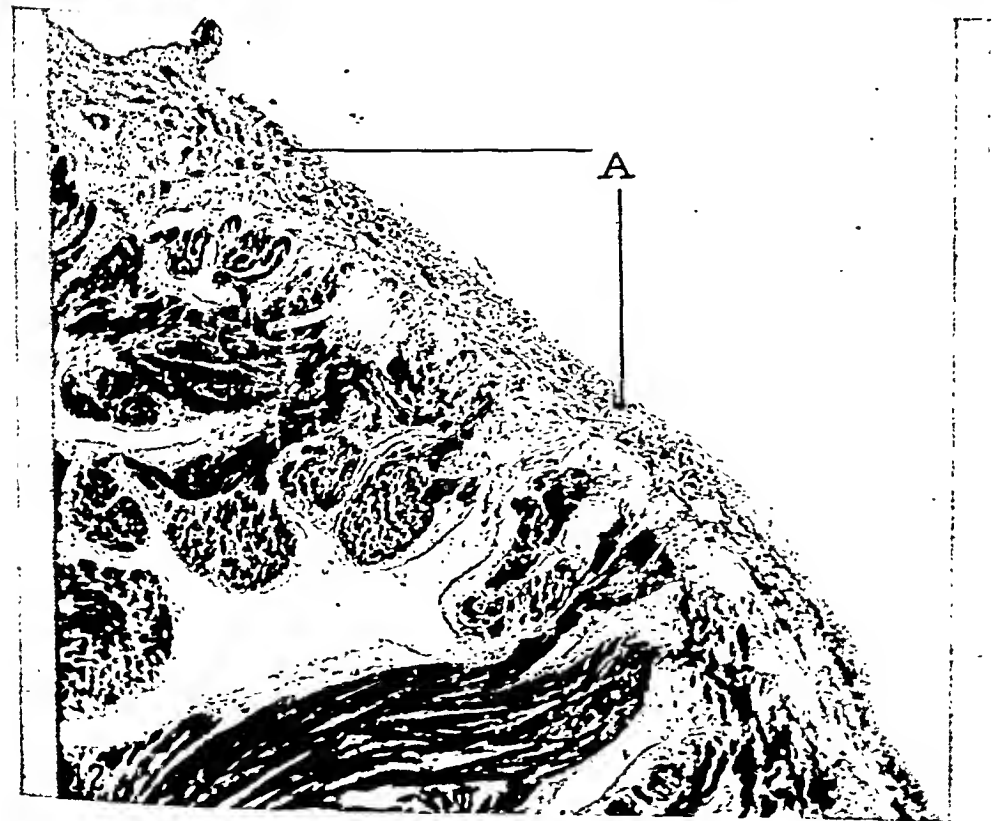
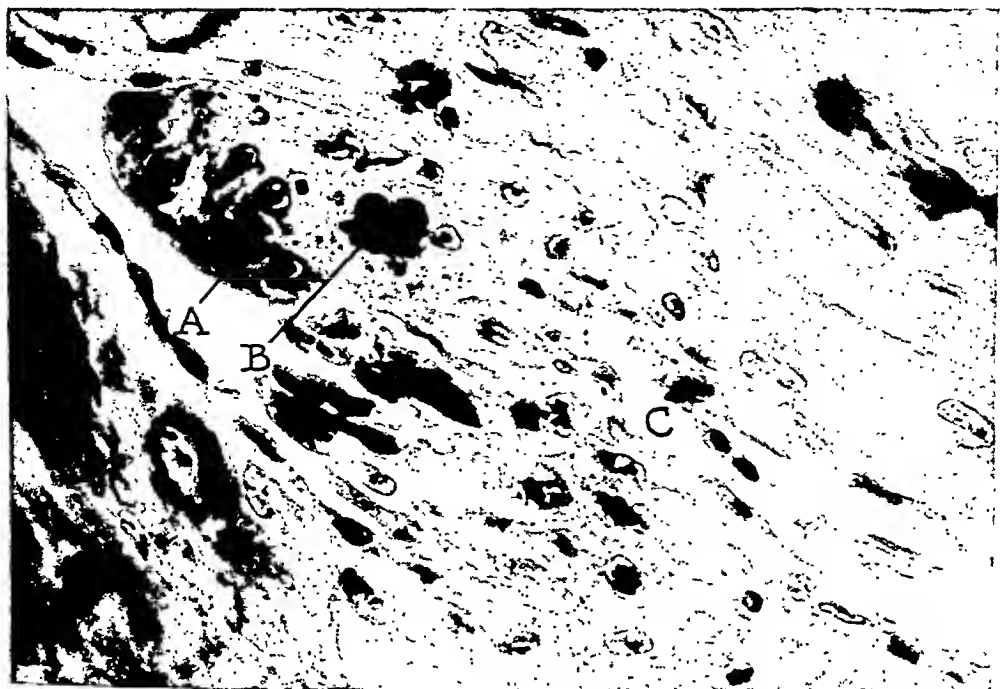


(Murphy and Swift: Induction of cardiac lesions)

PLATE 42

FIG. 11. Rabbit 70-58 (see Table I),—died 13 days after last of 8 infections; negative blood cultures 2 days prior to and at autopsy. Left ventricle; mosaic nodular granuloma arising from thin walled vein; granuloma cells lodged between frankly fibrinoid masses (*A*); cell at *B* has 2 nuclei; several cells with owl-eyed nuclei; axially arranged cells surrounding *C* have streamers of cytoplasm; dissolution of adjacent myofibers. Masson trichrome stain. $\times 659$.

FIG. 12. Rabbit 70-71,—sacrificed 16 days after last of 8 infections. Left auricle; *A*, epi- and subepicardial collagen converted into frankly fibrinoid material; Mallory aniline blue stain. $\times 127$.



(Murphy and Swift: Induction of cardiac lesions)

THE STRUCTURE OF ELASTIC TISSUE AS STUDIED WITH THE ELECTRON MICROSCOPE*

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PLATES 43 TO 47

(Received for publication, March 14, 1949)

In contrast to its near relative, collagen, elastic tissue has thus far received meager attention with respect to fine structure analysis. Both morphologically and chemically this tissue has in the past been generally considered to be a relatively homogeneous entity. The present study indicates this to be highly unlikely. At least two distinguishable entities have been observed.

Previous investigations revealed no distinctive patterns of structure either by x-ray diffraction (1, 8) or electron microscopy (17). Collagen, on the other hand, yielded x-ray patterns indicating a high degree of organization (1, 2, 9). A complex axial repeating period with considerable detail of intraperiod structure was demonstrated in the fibrils with the electron microscope (5, 13, 15).

Astbury (1) observed a typical collagen x-ray diffraction pattern in stretched ligamentum nuchae, which, however, was reduced to amorphous rings after autoclaving (to remove the large amount of collagen known to be present). W. J. Schmidt (12) described an increase in double refraction on stretching the ligamentum presumably due to orientation of fibrous units.

Wolpers (17) found it necessary to treat elastic tissue with pepsin in acid for 24 hours before he could obtain fibers of a size suitable for study with the electron microscope. This partially digested elastin¹ from ligamentum nuchae revealed large, branching, amorphous fibers varying in width from 2,500 Å to 200 Å, the very smallest seen being 80 Å. No axial periodicity was noted. Fixation in osmic acid resulted in a fine longitudinal fibrillation which Wolpers ascribed to the action of the fixative and not intrinsic to the actual structure. Orcein, a stain considered relatively specific for elastic fibers, was observed to deposit on these fibers in the form of small flakes. The elastin of the mouse aorta, after acid pepsin digestion, osmic acid fixation, and sonic fragmentation, appeared as thin, fenestrated laminae with numerous, short, stubby fibers protruding from the surface.

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¹ The term "elastin" will be used synonymously with elastic tissue in this report and will not define a particular protein.

Meyer and Ferri (11) from thermoelastic considerations, deduced that elastin is probably made up of highly contorted polymeric chains bonded laterally by only few links. Stretching the fiber orients these units in an axial direction thereby reducing the entropy and increasing the internal energy. Thermal motion produces the elastic recoil. Jordan Lloyd (7) agreed with this interpretation and added that the structure differs from rubber in that the peptide linkages of the elastin polymeric chains have polar properties which, by cross-linking, serve to maintain the extended state in stretched and dried elastic fibers. Imbibition of water is necessary to spread the chains apart, thus permitting thermal contraction whereas rubber, being entirely apolar, will not "lock" in the extended state when dry at ordinary temperature. McCartney (10) observed that ligamentum nuchae when freed of its collagen by peptic digestion contracts linearly with increase in temperature and returns along the same straight line to its original length on cooling. This suggests no extensive alteration in structure when the tissue is heated to 100°C. in water.

Chemical analysis of ligamentum nuchae by Stein and Miller (16) indicated roughly one in thirty amino acid residues to be polar (excluding glycine) in contrast to about 50 per cent for collagen.

Methods

The swim bladder of the carp, the aorta of the adult rabbit, and 2 day old rat, that from a human being 5 years old, and ligamentum nuchae of the cow were used as sources of elastin in this study. The swim bladder² proved to be a particularly suitable source of material because the collagen (ichthyocol) was readily removed by heating to 60°C. in dilute acetic acid, although nearly all of it could be removed by the dilute acid without heat. Mild fragmentation procedures reduced the elastin fibers to suitable size. Elastic tissue of the ligament and aorta posed difficult problems of fragmentation.

Metal shadowing was found to be the most suitable preparative adjuvant although heavy metal staining (6) was also attempted. The same preparative procedures were used for all tissues except for modifications required by certain special characteristics of each material.

The elastin of the carp swim bladder was prepared in the following manner. The tunic was stripped from the fresh bladder, washed well with water, cut into small pieces, and allowed to stand overnight in 1 per cent acetic acid in the refrigerator. The collagen swells greatly forming a viscous solution which is readily filtered off through silk bolting cloth. The gelatinous residue was then placed in dilute acetic acid and fragmented for 10 seconds in a Waring blender. The suspension, which is fairly viscous, was centrifuged to sediment the finely dispersed elastin. Repeated washing in the centrifuge in dilute acid removed practically all the "dissolved" collagen, leaving a residue which formed a fine, cloudy suspension on agitation. However, to be certain that no collagen filaments (14) were present to confuse the picture, the suspension was boiled for 30 minutes. Swim bladder collagen is readily gelatinized at this temperature. Heating greatly reduces the viscosity of the suspension and facilitates sedimentation of the elastin fibers. As will be described later, heat and dilute acid do not seem to affect the observed structure of intact elastin. The finely comminuted elastin was then washed several times in the centrifuge and resuspended in distilled water.

The aortic tissues and bovine ligamentum nuchae were prepared in a somewhat different fashion. The fresh, unfixed tissues were sectioned with the freezing microtome. One portion was boiled for 1 hour in 1 per cent acetic acid to remove the collagen, while a second portion was not heated so as to permit study of the relationship between collagen and elastin.

² Faure-Fremiet and Garrault (4) give an excellent histological description of the fish swim bladder.

In order to reduce the elastic fibers to a size suitable for electron microscopy preparations were further fragmented in a sonic (9 kc. magnetostriction type) oscillator.

The resulting suspensions were prepared for examination in the usual manner by depositing drops on supporting films of conventional nickel specimen grids and blotting them off with filter paper after about 2 minutes. Some preparations were examined without further treatment. Most were shadowed with 8 mg. of chromium or 10 mg. of uranium at an angle of 10° and a filament to specimen distance of 20 cm.

The effect of digestion with crystalline trypsin (Armour) was studied by incubating portions of each material, boiled and not heated, in 0.1 per cent enzyme buffered to pH 8-8.4 with NaHCO_3 at 37°C . for periods ranging from $2\frac{1}{2}$ to 24 hours under toluene. These suspensions were then washed in the refrigerated centrifuge at 4,000 R.P.M. for 1 hour with distilled water to remove the dissolved solids. Specimens were prepared for electron microscopy as described, except that the drops were allowed to remain on the grids for at least 10 minutes before blotting. Buffered controls without enzyme were also examined.

The influence of temperature on the fibrous elements released by tryptic digestion was investigated by heating portions of the washed, trypsinized suspensions in a water bath at temperature intervals of about 10°C . ranging from 40° to 100° for 30 minutes.

Pure crystalline trypsin solutions were examined to rule out possible artifacts from this source.

The effect of pH on the washed, trypsinized suspensions was studied in the range 2.5 to 10.5 at nearly unit intervals and room temperature. Unbuffered acetic acid and ammonium hydroxide were used to make up the pH series. This experiment was not performed on the rat aorta.

To observe the effect of formalin fixation portions of boiled ligamentum nuchae elastic tissues were fixed in 10 per cent neutral formalin for 7 days, incubated in trypsin, and prepared for electron microscopy as described.

An RCA type EMU electron microscope was used in this study.

RESULTS

Tissue of the Fish Swim Bladder.—The elastic tissue of the swim bladder tunic is extremely friable, requiring only 10 seconds in the Waring blender to reduce it to a fine, cloudy suspension. Electron microscopy revealed the suspended particles to be long, contorted, branching fibers ranging from about 300 A to 5,000 A in width and often measuring many microns in length. In addition to the fibers there were numerous clumps of coarse, granular material. Excessive blending destroyed most of the fibers leaving only granular masses. In some preparations which had not been heated but only washed in dilute acetic acid, extremely fine filaments were present in the background which disappeared on heating to 40°C .; these were most likely ichthyocol (14). No intact collagen fibrils were found in boiled preparations. The following descriptions are based on electron micrographs of chromium-shadowed specimens.

The fibers appeared to be irregularly flattened on the supporting film (possibly a drying effect) and usually showed a roughly parallel and often coarsely interlaced fibrillation. These fibrillar units ranged in width from about 300 A to 1,000 A and appeared to be embedded in an amorphous matrix. The latter was observed to flatten out at the edges and often assumed a finely packed texture (Fig. 1). The "grain" of this texture, which was not always observ-

able, ran both parallel and perpendicular to the long axis in different fibers. Branching of a fiber was characterized by a separation of the fibrils (see Text-fig. 1) with a stretching and thinning of the binding matrix at the bifurcation. Occasionally a frayed fiber would show the presence of fibrous units no more than 100 A. in width. There was no evidence of a true axial periodicity.

Boiling for 30 minutes in 1 per cent acetic acid had no effect on the observable structure.

Observations of unshadowed fibers added nothing to the pattern and the limited number of experiments with osmic and phosphotungstic acid staining were unproductive.

Aorta of the Adult Rabbit, Rat, and Human Being.—Boiling frozen sections of aorta in 1 per cent acetic acid destroys the collagen and most probably all other structural elements except the elastin. Fragmentation by freeze-sectioning and high speed homogenization produced very few tissue fragments small enough for electron microscopy. However, strong sonic vibrations disrupted the strongly coherent elastic membranes, producing fibers of size suitable for study (Fig. 4). These closely resembled the elastic fibers found in the tissue of the swim bladder. Whether the fibers observed were part of the lamina or actually represented the interlamellar fibers could not be determined. Considerable amounts of granular material were also present. No fine filaments were found. Large numbers of typically striated collagen fibrils were associated with the elastic fibers in the unheated preparations and often appeared to protrude from the fragments of elastin.

Ligamentum Nuchae of the Cow.—The long, thick fibers of this tissue were extremely resistant to fragmentation, more so than the fibers of the aortic elastica. Prolonged treatment with 9 kc. sonic waves was required to produce even a few suitable fibers. The fragments were usually small irregular chunks rather than fibrous units. Apparently these fibers are as strongly coherent in the lateral direction as they are longitudinally. The few fibers obtained were too thick and dense to reveal any detailed structure. In one rare case of a much flattened, frayed elastic fiber, numerous fine filaments about 100 A in width were found lying parallel with the fiber axis.

The Effect of Trypsin.—The most striking result of tryptic digestion was the appearance of many fine threads of constant cross-section in all fields examined and in preparations of all three tissue types. They were barely visible in unshadowed preparations. Threads released from the three different types of elastic tissue were indistinguishable with regard to their morphology (Figs. 6, 7, and 9).

In particularly clean preparations two morphological forms of threads were observed. One type found in varying numbers in different preparations of the same tissues was a tightly and evenly coiled double helix formed by the twining of two thin, apparently smooth filaments. In the same fields many uncoiled

filaments could be found usually lying in closely associated, parallel pairs and usually much longer than the coiled forms (Figs. 8 to 10). There were many instances observed in which a coiled thread was transformed abruptly into a parallel pair of smooth filaments, and in some cases, as shown in Fig. 7, only a single filament would project beyond the coil. Occasionally both ends of a coiled thread would continue as a pair of parallel filaments. The widths (actually height above the supporting film) of 350 filaments and threads obtained from ligamentum nuchae were determined from the shadow length, the only selection factor being the perpendicularity of the long axis of the thread to the direction of the shadow. A characteristic width of about 120 Å was calculated for the coiled threads and approximately 70 Å for the individual filaments. These figures apply as well to the threads and filaments obtained from the other tissues studied. Direct measurements of widths on an unshadowed preparation (which is not entirely satisfactory because of low contrast) roughly confirmed these figures.

The pitch of the helix in 120 measured threads from ligamentum nuchae ranged from 470 Å to 580 Å with a peak at about 530 Å. Again, these figures were approximated by those for the aortic threads. These fibrous elements were usually straight or gently curved but never acutely kinked. No discrete axial periodicity was resolved other than that produced by the observed coiling.

Threads and filaments were never found in preparations not digested by trypsin.

Effects of Temperature, Formalin Fixation, and pH.—Heating threads and filaments of each of the three forms of elastic tissue suspended in distilled water resulted in their disappearance in the temperature range 70–85°C. Boiling the tissue in dilute acid prior to tryptic digestion did not alter the critical temperature.

Formalin-fixed ligamentum nuchae which had been boiled in dilute acid prior to fixation was digested in trypsin in a manner identical with that described for the fresh material. Typical threads and filaments were found. No further characterization was attempted at this time.

The influence of pH on the state of aggregation and morphology of the threads obtained from ligamentum nuchae was studied. At pH 3.0 only amorphous clumps of material often resembling small, flat discs were observed. At about pH 3.6 these clumps were larger, flatter, more irregular, and more granular (Fig. 13). At pH 5.1 the aggregates appeared coarsely reticular with short, stubby, nodular threads protruding from the edges. No obvious coiling was observed but there seemed to be a fine, granular debris covering everything which obscured detail (Fig. 12). No free filaments were observed. At increasingly higher pH the threads were increasingly longer and finer. At pH 5.7 some free threads were observed, plus many loose aggregates. At pH 6.2 very little aggregation was observed and characteristic threads and filaments

were present. Coiled forms were found in large numbers in the pH range 6.2 to 9.1 (Figs. 7 and 8). If a suspension of threads were allowed to stand for about 24 hours at neutral pH, clumping was also observed.

The ratio of coiled threads to smooth filaments diminished with increasing alkalinity; at pH 9.8 no coiled threads could be found (Fig. 11). Increase in pH had no observable influence on the pitch of the helices; they were either coiled with the characteristic pitch or completely uncoiled. Boiling the tissue in dilute acid prior to digestion did not alter the pH effects. Less detailed experiments performed on the threads of rabbit aorta and fish swim bladder indicated a similar behavior pattern.

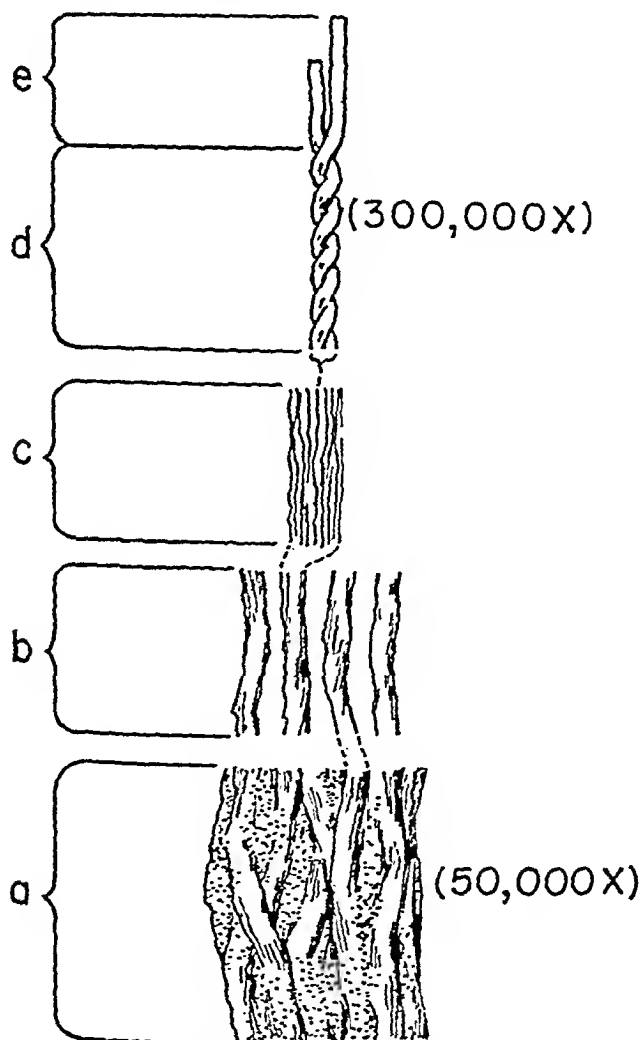
Partially Digested Fibers.—Trypsinized elastin of the swim bladder tunic and aorta revealed numerous fibers of a size well suited for electron microscopy. Many of the partially digested swim bladder fibers appeared to be flattened, amorphous, and moth-eaten (Fig. 3). In many cases, however, the amorphous matrix seemed to have been partially stripped away, revealing nearly naked fibrils (Figs. 2 and 5). In rare instances partial fragmentation of these fibrils revealed thinner units of about the size of the free threads. However, even here there seemed to be enough amorphous material present to obscure any details such as coiling. The characteristic coiled threads have not yet been observed in either intact or partially digested fibers.

DISCUSSION

From the data at hand one may construct a tentative model of the architecture of the elastic fiber (see Text-fig. 1). At least two distinct chemical and morphological components, namely the threads and the amorphous binding substance, are associated to form a fiber which is capable of long range elasticity, great mechanical strength, and refractiveness to boiling water, dilute acids, or alkalis. Collagen is present as an incidental component, probably incorporated in the fiber extracellularly during its formation as suggested in Bloom's study of elastic fibers in tissue culture (3). The threads are arranged in long, roughly parallel bundles, such as observed in partially digested fibers, which may or may not course the full length of the fiber. These bundles are probably both infiltrated with and embedded in the trypsin-sensitive, amorphous binding matrix. The relative proportions of matrix and threads cannot readily be determined by electron microscopy.

Matrix.—Whether this substance is amorphous in a rigorous sense or is actually a tangled mass of long macromolecular chains like rubber, remains to be determined. The matrix substance is apparently responsible for the heat resistance of the intact fiber since the naked threads (released by trypsin) are relatively heat-labile. The matrix is probably bound to the filaments in such a way as to prevent their destruction by heat. Probably the great difference in friability between the intact fibers of the different elastic tissues is determined by the tensile strength of the matrix.

It is interesting to note that formalin fixation apparently does not prevent the digestion of the matrix material by trypsin. This fact may be of significance in considering the chemical nature of the matrix.



TEXT-FIG. 1. Schematic representation of the structure of the elastic fiber (collagen fibrils omitted). (a) Undigested fiber showing fibrils imbedded in amorphous matrix (see Fig. 4). (b) Partially digested fiber showing fibrils stripped of matrix (see Figs. 2 and 5). (c) Single fibril, enlarged, revealing it to be a bundle of threads. (d) Single coiled thread, greatly enlarged (see Figs. 8 and 9). (e) Individual filaments which, when twined, form the thread (see Figs. 10 to 11).

The Fibrous Component.—The threads obtained from the different elastic tissues are similar in many respects; e.g., morphology, heat sensitivity, and

reaction to pH. This suggests that these characteristic units are a uniform constituent of elastic tissue.

The tendency to aggregate on the acid side and to fray into finer threads at higher pH suggests an isoelectric point in the acid range. Stein and Miller (16) from electrophoretic studies of purified, finely comminuted, whole elastic tissue estimated an isoelectric point of 4.8. At the present time there are no further chemical data to characterize these structures more fully.

The coiling of these fine fibrous elements is of great interest because of its possible rôle in elastic and contractile processes. Indeed, the structural pattern described here has all the components of the molecular model for elastin postulated from thermodynamic considerations by Meyer and Ferri (11); *i.e.*, a compressed, coiled spring held under tension by a stretched elastic band. If this analogy were pursued, one would expect that the observed coiled threads are compressed in the native state and that tryptic digestion frees them from the restraining force of the matrix, thus permitting a large increase in pitch. It is therefore important to demonstrate conclusively whether the coiled structure is characteristic of the intact tissue and not a result of manipulation. The chance that the coiled threads are artifacts produced by the axial rotation of adjacent filaments while in suspension is remote. The coils are always tight and regular, and show little variation in pitch even in different forms of elastic tissue; moreover intermediary stages of coiling have never been observed. Another bit of evidence is the appearance of large numbers of filaments and the disappearance of coiled threads with a rise in pH and the inability to reunite these threads into coils on lowering the pH. Only haphazard lateral aggregation with some irregular twining occurs. The assumption here is that the smooth, thin filaments are produced by uncoiling of the threads and are not a separate species.

The large numbers of parallel pairs of filaments which sometimes abruptly twine to form a short segment of typically coiled thread suggest that two filaments form a single thread. Because the individual uncoiled filaments are nearly always very much longer than the coiled threads—longer than one would expect to result from simple uncoiling of the thread—there exists the possibility of a much finer, molecular coiling in the filament which is also smoothed out when the thread uncoils. It is evident, however, that considerably more data are needed to answer conclusively the basic questions as to the morphology of the threads in the native state and the respective rôles played by the two components in elastic behavior.

Further experimentation with the electron microscope and physical chemical techniques on the liberated threads, and also x-ray diffraction and polarized light studies on the intact tissue, are needed to provide the essential evidence. With regard to early polarized light studies (12) the correlation between the observed isotropy of relaxed elastic tissue and the structures described here is obscure at present.

Stein and Miller (16) have made an amino acid analysis of "purified" elastin. However, they have treated this substance as a single homogeneous protein even to the extent of working out the amino acid "frequencies" according to the Bergmann-Niemann "periodicity theory." The results described here demonstrate the presence of at least two components. It is therefore highly desirable that new amino acid analyses be made on the individual components. This should now be feasible since the present work indicates procedures by which the two fractions may be separated.

The author wishes to thank Mrs. Mary Frances Simmons and Mr. James Wilson for their valuable technical assistance.

CONCLUSIONS

Electron microscope examination of fragmented elastic tissue obtained from fish swim bladder, bovine ligamentum nuchae, and aortas of various mammals, including man, reveals characteristically formed fibers and much amorphous material. Boiling in dilute acid destroys the associated collagen but does not obviously alter the elastic tissue.

Digestion in crystalline trypsin of either boiled or unheated tissue from any of the above-mentioned sources causes the release of thin threads ranging in length from 0.1μ to many microns. A large proportion of these threads are evenly and tightly coiled double helices formed from at least two interlacing filaments and measuring about 120 A in width. The distance between coils ranges from about 470 to 590 A. The individual smooth filaments, many of which are present in parallel pairs, measure approximately 70 A in width.

Raising the pH of a neutral suspension of threads from ligamentum nuchae lowers the ratio of helical threads to uncoiled filaments, whereas lowering the pH with acetic acid results in clumping of threads with complete loss of identity at about pH 3.6.

Threads and filaments obtained from all sources studied were destroyed in the temperature range 75–85°C. at pH 7.

It is concluded that the elastic fiber is a two component system composed of bundles of trypsin-resistant threads of characteristic form and size plus a trypsin-sensitive, heat-resistant "amorphous" binding matrix.

The possible relationship of this structure to the elastic properties of the tissue is discussed.

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EXPLANATION OF PLATES

PLATE 43

Fish swim bladder elastin heated at 60°C. in dilute acid. Fragmented and washed. All specimens were chromium-shadowed.

FIG. 1. Fibers deposited on grid from aqueous suspension. $\times 14,000$.

FIG. 2. Fiber which has been partially digested with crystalline trypsin. Much of the amorphous matrix has been removed revealing the fibrils. $\times 32,000$.

FIG. 3. After digestion with trypsin filaments released by the enzyme can be seen. An amorphous fragment of a partially digested fiber remains. $\times 31,000$.



PLATE 44

Adult rabbit aorta boiled in 1.0 per cent acetic acid, freeze-sectioned. All preparations were chromium-shadowed. $\times 22,000$.

FIG. 4. Fiber from suspension produced by treating tissue sections with sonic oscillations. Coarse fibrillation of the structure is noted, plus considerable amounts of amorphous material. $\times 22,000$.

FIG. 5. Partial tryptic digestion of an aortic elastin fiber, revealing the roughly parallel fibrils which are believed to be bundles of threads. Many free threads can be seen in the background. $\times 21,000$.

FIG. 6. Aortic elastin threads released by trypsin. Characteristically coiled threads can be seen in addition to some uncoiled filaments. The large, dense lobular mass is an unidentifiable contamination. $\times 28,000$.

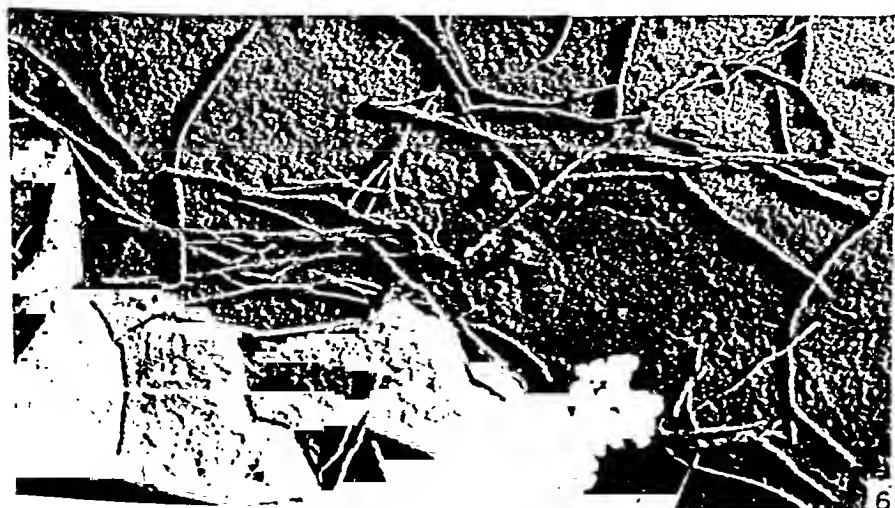
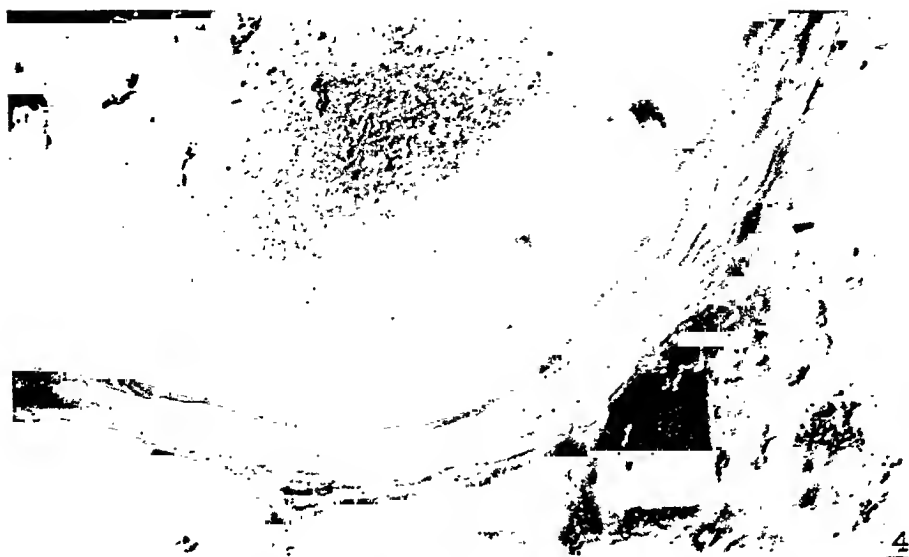


PLATE 45

FIG. 7. Threads and filaments released from bovine ligamentum nuchae by tryptic digestion. Numerous coiled threads are observed, along with thinner uncoiled filaments which in some cases can be seen to project individually beyond the coiled regions. Shadowed with chromium. $\times 23,000$.

FIG. 8. Higher magnification of coiled threads and filaments. Shadowed with chromium. $\times 42,000$.



(Gross: Structure of elastic tissue)

PLATE 46

FIG. 9. Threads and filaments released by trypsin from the elastin of the fish swim bladder tunic. A single coiled thread is seen in this field. The filaments are much longer than the threads and in most cases are paired. Shadowed with chromium. $\times 29,200$.

FIG. 10. Human aorta from 5 year old girl. Not preheated. Digested with trypsin. pH raised to 10.0. Thin, uncoiled filaments are observed. Coiled threads have not thus far been found at this pH. Two typical intact collagen fibrils are also present. Shadowed with uranium. Pebbly background represents unusually coarse collodion film structure. $\times 36,100$.

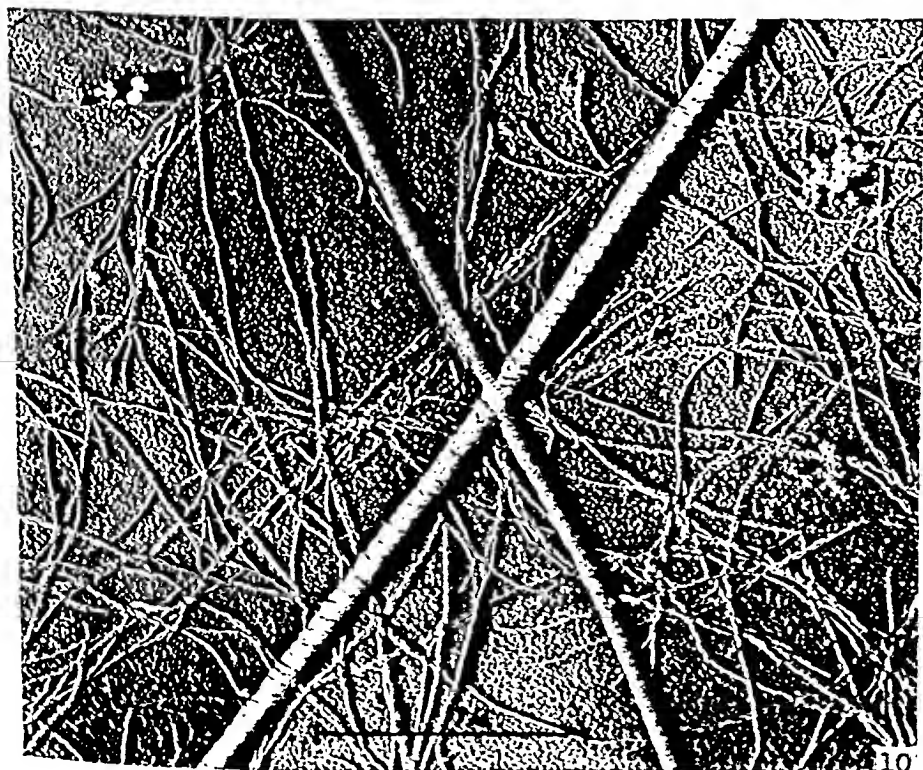
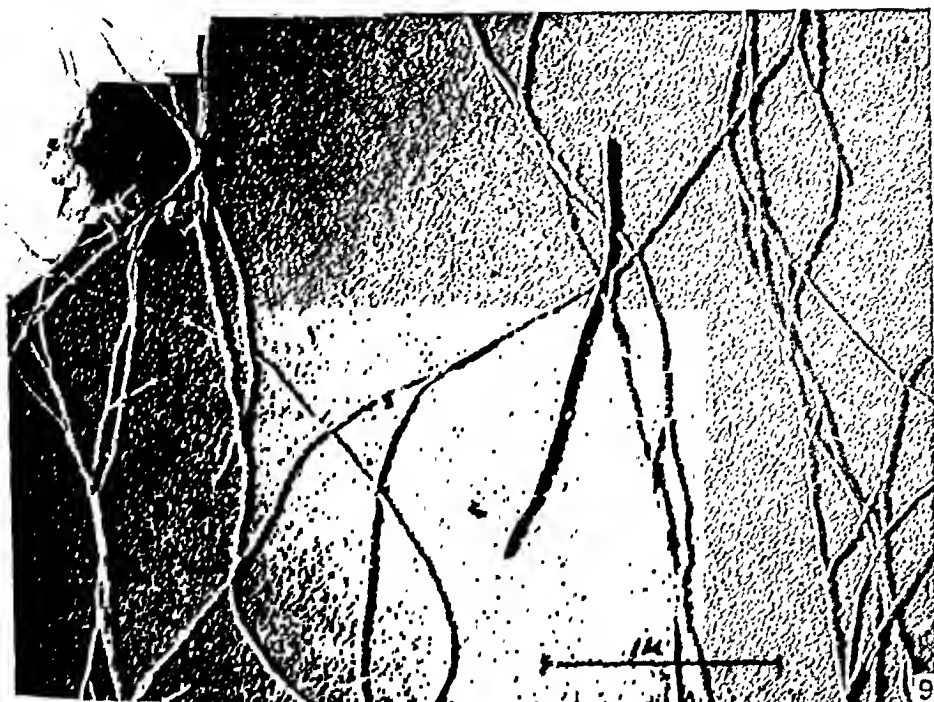


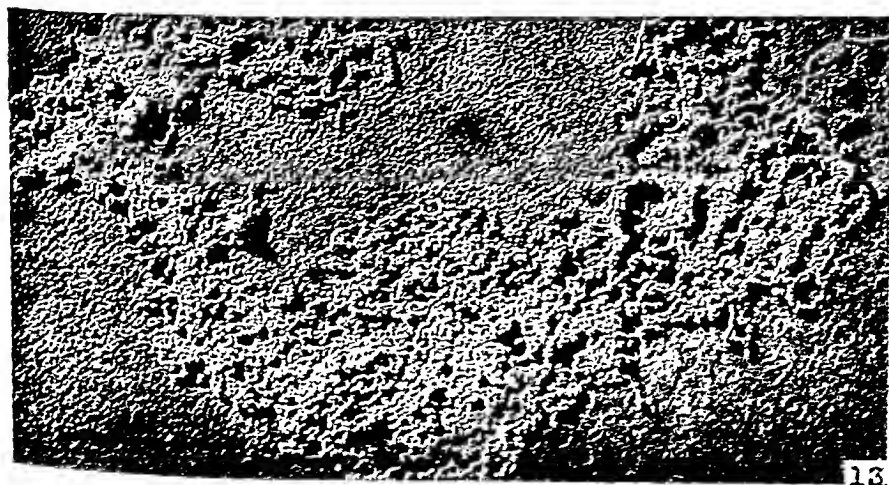
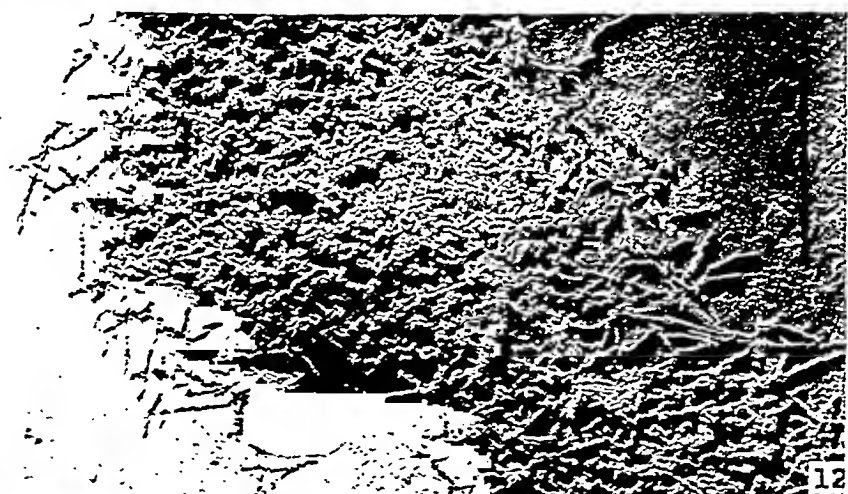
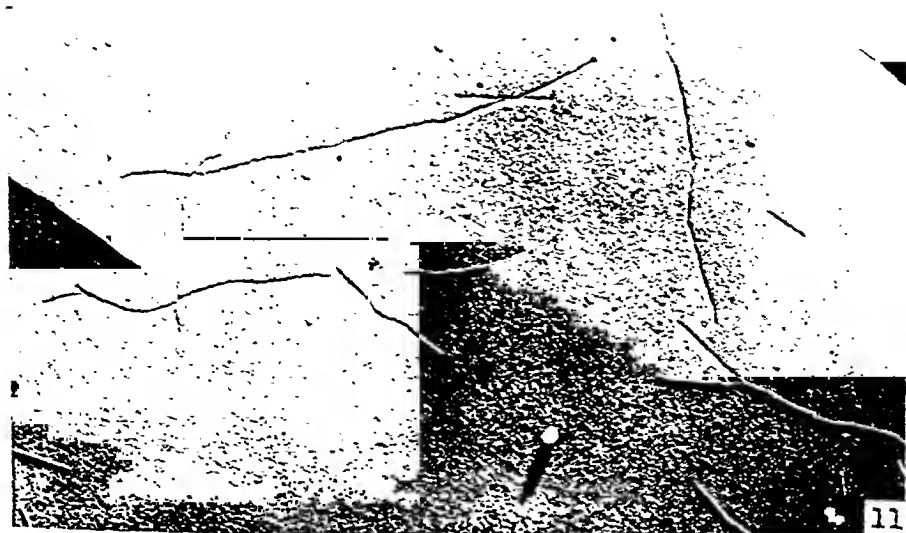
PLATE 47

Influence of pH on ligamentum nuchae threads. All specimens were chromium-shadowed.

FIG. 11. pH 9.8. Only individual and occasionally paired filaments are observed. No coiled threads were seen. $\times 22,500$.

FIG. 12. pH 5.1. Clump of short, irregularly nodular threads. Coiling was not resolved. $\times 24,750$.

FIG. 13. pH 3.6. Granular mass in which individual threads are no longer identifiable. $\times 21,750$.



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